Physiological Zoölogy

Vol. XIII OCTOBER 1940 *No. 4*

THE PRIMARY DEVELOPMENT OF THE SKELETON IN NERVELESS AND POORLY INNERVATED LIMB TRANSPLANTS OF CHICK EMBRYOS[1]

(Three figures and two plates)

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THE nervous system participates in several indirect ways as a factor in the development of the skeleton. It has a trophic influence on the developing limb bud, including its skeleton. This was shown for the frog, where the noninnervated but otherwise undisturbed hind limb exhibits a growth reduction of about 12 per cent (Hamburger, 1928). A similar effect, though less clearly analyzed, seems likely to exist in the case of the chick limbs. The most obvious indirect action of the nervous system would be by way of excitation of muscular activity. The question of whether functional movement of adjacent elements is a factor in molding and shaping the cartilages and bones and particularly the articulating surfaces and in preventing fusions of joints has been widely discussed and is still controversial. The experimental analysis of these problems has been ably reviewed by Murray and Selby (1930) and by Murray (1936). They arrive at the conclusion that, whereas the gross form of the individual skeletal elements is attained by self-differentiation from early stages on, such extrinsic factors as outlined above are essential in perfecting and refining the details and in forming functional joints.

These conclusions are based largely on experiments on the limb skeleton of the chick, using chorio-allantoic grafts and tissue-culture experiments as methods of analysis. Our method of transplanting limb primordia to the flank or into the coelomic cavity has certain advantages over the other methods. It provides for optimal conditions as far as nutrition and space requirements for the developing limb are concerned. Furthermore, very young stages of limb primordia may be transplanted, and the observations may be extended over a long period of time, possibly even beyond hatching. In an extensive series of such transplants (Hamburger, 1939) a number of normally shaped, nerveless, or poorly innervated limbs were obtained, the skeletons of which were studied in detail. The present investigation is limited to the differentiation of the cartilaginous skeleton and the early phases of osteogenesis.

[1] This investigation was aided by a grant of the Rockefeller Foundation. The authors are indebted to Miss Marie Jakus for the preparation of the drawings and to Mr. E. L. Keefe for the preparation of the photographs.

MATERIALS AND METHODS

Wing and leg primordia of chick embryos of 51–72 hours of incubation were transplanted heterotopically to hosts of the same stages. For details of technique and seriation of stages see Hamburger (1938, 1939). Forty-nine normal and 27 malformed cases were selected for the present investigation. The latter are included in order to study the type and range of skeletal abnormalities resulting from transplantation. Atypical short outgrowths which have been obtained mainly from late transplanted limb buds (stages 5 and 6) are omitted. The transplants are fixed at an age of 9–21 days, most at the age of 9–10 days.

Fourteen cases (5 wings, 9 legs) were sectioned, stained with iron hematoxylin, and their nervous and skeletal systems reconstructed. Fifty-four cases (29 wings, 25 legs) were stained *in toto* with methylene blue, following Lundvall's technique, which is a differential stain for cartilage.

The method used by us is briefly as follows: The whole embryos are stained in a solution of methylene blue in 70 per cent alcohol (0.25 gm. per 100 cc. of alcohol) for 48 hours, destained in acid alcohol for 2–3 hours, washed and run up through the alcohols (70 per cent-95 per cent-absolute), cleared in a solution of equal parts of absolute alcohol and benzene for 6 hours, and then in pure benzene for 12 hours. They are hardened and stored in a solution of three parts oil of wintergreen to one part benzyl benzoate, according to the Spalteholz method.

Two legs (21 days) were stained differentially for bone with alizarin red, and 6 transplants (2 wings, 4 legs; 18 days and older) were dissected.

THE INNERVATION OF THE TRANSPLANTS

The innervation of transplanted limbs has been described in detail (Hamburger, 1939). It is found that only transplants located near the spinal cord receive an appreciable amount of nerve supply, whereas transplants located ventrally or inside the coelomic cavity are very poorly innervated or nerveless. The quantitative development of all transplant nerves is surprisingly poor. Movements of transplants are observed occasionally but are never extensive. They are found only in transplants located near a host limb and supplied, at least in part, by limb nerves. Absence of visible motion, however, does not exclude the possibility that individual muscles may have been functional.

Of the present material, 4 wings and 5 legs are definitely nerveless, as ascertained in sections or by dissecting. These are located in the coelom or loosely attached to the mesenteries of the umbilical cord. They are, then, the "crucial" cases. The innervation of the cases stained in methylene blue and alizarin red was not observed directly. Most of them are located in the ventral body wall, in the coelom, or in the umbilical cord. Their innervation must be absent or, at best, very poor. Even though innervated, they would not be capable of extensive motility, because almost all transplant girdles are very poorly developed (see below) and are imbedded in the soft tissues of the host. Of the cases used in the present study only 4 wing transplants implanted in the place of, or near to, the host wing have received an appreciable nerve supply.

MORPHOGENESIS

Free appendages.—Table 1 shows that 49 per cent of the free appendages are complete and normal (see Figs. 1, 5, 6, 7). In 12 other cases, only 1 phalanx is missing, so that 49 cases (23 wings and 26 legs) may be considered as typical differentiations. The pattern of

carpals and tarsals is not considered in detail; they are found to be normal in numerous instances. Since 6 of the 9 "crucial" cases belong in this category, it is conclusively shown that the formation of a typical pattern of skeletal elements and their gross morphogenesis are independent of any indirect influence of the nervous system. This statement must be qualified with regard to joint formation (see p. 373).

Hypodactyly is found in 7 cases. In 6 of them it affects the first digits, which are the shortest in normal wings and legs (Fig. 4). In 2 cases the hypodactyly is combined with a slight hypophalangy on the digits present. The fibula, which is rudimentary in normal legs, is absent in 9 cases, in 8 of which the deficiency is combined with hypodactyly. This hypodactyly concerns the digits on the fibular side in some cases and the digits on the tibial side in others. Nine cases show more severe malformations. It is of interest to note that the radius and ulna, or the tibia and fibula, are here most seriously affected; they are abnormally short and atypical in shape. On the other hand, the femur is always normal, and the humerus is normal in all but 2 cases. These defects may or may not be combined with hypodactyly and hypophalangy. In several cases the long bones are abnormally curved, the metacarpals or the metatarsals, respectively, being affected most frequently. One of the malformed legs resembles the chondrodystrophic condition which is described for the chick in a nonhereditary and a hereditary form by Landauer (1927, 1931). The femur is normal and straight; the tibia is extremely curved and shows hypertrophy of bone on the concave side; the fibula is abnormally strong and of the length of the tibia; both tibia and fibula are fused at their two ends; and one metatarsal bone is curved. A survey of our complete material shows that, although transplantation may interfere with morphogenesis, the resulting malformations are ordinarily not of this type. It seems likely, therefore, that the donor embryo of this particular transplant was incidentally chondrodystrophic.

TABLE 1

MORPHOGENESIS OF TRANSPLANTED LIMB SKELETONS

Condition	Wings	Legs	Total	Percentage
Complete and normal	17	20	37	49
Complete, except absence of one phalanx	6	6	12	16
Hypodactyly	4	3	7	9
Absence of radius or fibula, otherwise normal	1	1	2	2
Absence of fibula or ulna, combined with hypodactyly	1	8	9	12
Malformations of higher degree	7	2	9	12
Totals	36	40	76	100

Duplications are rare. They concern the distal parts only; we find duplications of 1 or 2 phalanges in 3 cases, and of metacarpals and phalanges in 2 cases. One of the latter duplications is in a host wing, whose development apparently has been interfered with by the transplant.

It is obvious that the distal parts are more frequently affected than the proximal parts, and a definite sequence in the deficiencies in distal-proximal direction is observed. Distal

elements, particularly the phalanges, may be abnormal or absent in the presence of normal proximal parts, but absence or abnormality of the latter is generally correlated with defects in the distal parts. No such regularity is found, however, in the pre-postaxial direction. Preaxial elements are affected alone or together with postaxial elements, and vice versa. The radius or tibia may be imperfect or absent in the presence of its normal partner, and vice versa. We conclude that from the stage of transplantation of the bud onward, there are no reciprocal causal relations between preaxial and postaxial elements, nor is there a causal subordination of both to a dominant factor.

The causation of the malformations is obscure. The following factors are excluded as causal agents: stage of the limb buds at transplantation, the site of the transplant (whether in the flank or in the coelom), the axial orientation of the transplant, and the innervation of the transplant. In a few instances concerning purely postaxial deficiencies, the defect may be explained on the assumption that the bud was cut short on its posterior edge. The majority of distal anomalies, however, cannot be explained in a similar way. In a few instances a host limb, developing immediately adjacent to the transplant, interfered with the development of the latter. The malformations must be considered as an inhibition of development resulting from general nonspecific factors which are connected with the operation (such as delayed or insufficient blood supply, etc.).

Pectoral girdles.—Since methylene blue is a cartilage stain, the clavicle is not stained. No data on it are available from our experiments. It is found that in all cases but one the girdle is present. Both scapula and coracoid are present in 32 cases (Figs. 5, 7*b*), the scapula being absent in the remaining 3 cases. In practically every specimen, however, these girdles are reduced greatly, both in absolute size and in proportion to the transplanted limb; in most cases they are "miniature" girdles (cf. Figs. 7*a* and 7*b*). Actual measurements are given below (p. 372).

The two elements of the pectoral girdle are recognizable in every case, although abnormalities in shape are very common and much more frequent than in the free limbs. The scapula is affected in relatively more cases than the coracoid. In most cases, the scapula and coracoid join in the formation of a glenoid cavity, and they meet at the typical angle. In cases in which the transplanted girdle is located near the host sternum, its coracoid articulates with the latter. In several cases in which host and transplant girdles are lying close together, elements of the transplant are fused with those of the host or cartilaginous bridges connect the two. It is found that in several cases one of the two elements is abnormal in shape, while the other is normal. It seems as if each element is self-differentiating with respect to the other, from the time of transplantation on.

Pelvic girdles.—Complete girdles are present in 29 of a total of 40 cases (Figs. 1*b*, 4, 6). At least 2 elements are present in every case. The ischium is absent in only 1 instance, the pubis is absent in 2 cases, and the ilium is absent in 8 cases. The reduction in size and the abnormality in shape of the elements are even more marked than in the pectoral girdle (cf. Fig. 1, *a* and *b*, also Fig. 4). The ilium is most highly affected; in many cases, it is a normally shaped, flat, broad plate, extending anteriorly and posteriorly from the acetabulum, but often its outline is atypical. In some cases it does not extend beyond the acetabulum. The ischium is occasionally a narrow, rodlike structure rather than a broad, flat plate. The pubis is found to be more normal in size and structure than the other 2 elements. All 3 elements join in the formation of the acetabulum and are found in a typical position relative to one another. Fusions with adjacent parts of the host skeleton are very infrequent (in Fig. 6, the ilia of host and transplant are fused). The 3 elements vary

independently of one another with respect to presence or shape. No causal relationship seems to exist in their differentiation; nor do we find a correlation between the degree of differentiation in the free limb and the girdle.

It is difficult to interpret the size reduction and abnormal shape of the girdles,[2] since very little information is available concerning the localization of the girdle-forming primordia in the stages of operation. At least part of the material for all elements must be located in the transplanted limb primordium, i.e., in the somatopleure lateral to the somites (somites and entoderm are excluded from the transplant). The deficiencies may be explained in one of the following ways: Girdle material may have been destroyed at operation, or part of the girdle-forming area may be located outside of the transplant, and no adequate regulation has taken place. Warren (1934) in experiments of removal of the anterior or posterior half of the wing bud shows that girdle elements are at least partly located in the wing bud proper and that no regulation takes place. His operations, however, were made on limb buds of more advanced stages. We have definite information for one element, the ilium. Its primordium is located partly in the transplant and partly in embryonic structures median to it. In a few cases the donors of leg transplants were raised; they contain fragments of the ilia in their sacral regions; and the corresponding transplants likewise contain deficient ilia. A similar observation was made by Spurling (1923). Measurements support this contention; the ilia are disproportionately small as compared to ischia and elements of the free leg. The same holds for the average size of the transplanted scapulae as compared to the coracoids which are reduced in proportion to the free wings. This indicates that part of the scapula material is located outside of the wing bud proper. Another factor may have an adverse effect on girdle formation: most of the girdles, in contrast to the free limbs, develop inside of structures of the host body and may be exposed to pressure by neighboring tissues.

GROWTH

Altogether, 14 free wing and 9 leg transplants are exactly or nearly exactly of the same size as the corresponding limbs of the host embryo. The great majority of transplants are, however, smaller than the host limbs. In order to obtain precise figures for the growth relations the greatest lengths of the different elements of the girdles and of the free limbs are measured in transplants and in host limbs, and the measurements of the transplants compared with those of their own host, and the ratios then averaged. Altogether, 32 wings and 26 legs are used; the malformed transplants are excluded. The observations include transplants ranging from 8 to 17 days of incubation. No significant differences in relative length are found at different age levels.

Table 2 shows that most elements are reduced 20–30 per cent, whereas the dorsal girdle elements (scapula and ilium) are reduced over 50 per cent. The data indicate that in the wing the metacarpals are more highly affected than other elements, while in the leg the tibia is most highly affected. Our data, however, are not sufficient to establish definitely a differential growth inhibition of these elements.

Ratios within the transplants.—The excessive shortness of scapula and ilium finds its expression in the ratios of scapula:humerus; scapula:coracoid; ilium:femur; and ilium:ischium which are computed for each transplant and its host and then averaged (Table 3). Finally, the proportions of the proximal elements within the free limbs are calculated

[2] The illustrations of Murray (1926) and of Hunt (1932) show the same deficiency in girdle development in chorio-allantoic grafts.

for transplants and for hosts. The average ratio of humerus:radius is 1.01 for the host wings and 1.16 for the transplant wings, which is not a significant difference. The average ratio of femur:tibia is 0.79 for the host leg and 0.99 for the transplant, which emphasizes again the considerable shortening of the tibia.

The growth reduction of transplants may be due either to an inadequate establishment of vascularization of the transplants or to deficiencies in the innervation. The latter may act in a twofold way, either by controlling the contraction and expansion of blood vessels or by exerting a direct trophic effect on the growing limb bud and on its innervated structures. Two of the wings and one of the legs which had reached the full size of their host limbs are located in a position in which they cannot have received adequate innervation. To judge from these cases, vascularization rather than innervation seems to be the decisive factor in growth control of transplants.

TABLE 2

COMPARISON OF LENGTH RATIOS OF TRANSPLANT AND HOST ELEMENTS

Trpl./Host	Hum./Hum.	Rad./Rad.	Metacar./Metacar.	Scap./Scap.	Cor./Cor.	Fem./Fem.	Tib./Tib.	Metatar./Metatar.	Il./Il.	Isch./Isch.
Averages.....	0.78	0.77	0.71	0.42	0.71	0.80	0.70	0.80	0.46	0.61

TABLE 3

COMPARISON OF LENGTH RATIOS WITHIN THE HOST AND TRANSPLANT LIMBS

	Scap./Hum.	Scap./Cor.	Hum./Rad.	Il./Fem.	Il./Isch.	Fem./Tib.
Host ratio.....	1.04	1.54	1.01	1.21	1.94	0.79
Transplant ratio.....	0.60	1.08	1.16	0.70	1.62	0.99

On the other hand, observations on nerveless frog limbs (Hamburger, 1928) point to growth control by the nervous system. In these experiments the lumbrosacral section of the spinal cord was extirpated unilaterally at early stages prior to nerve outgrowth, whereas the limb bud was not disturbed at all. In these cases the ensuing growth reduction of about 12 per cent must be due to a direct or an indirect effect of the lack of innervation, since the operation does not interfere with the vascularization of the limbs.

HISTOLOGICAL DIFFERENTIATION OF CARTILAGE, AND EARLY OSSIFICATION

Chondrogenesis and early osteogenesis of the transplants are found to be absolutely typical and to proceed synchronously with the host limbs. The arrangement of the cartilage cells in the epiphyses and diaphyses (so-called "functional structures") and the formation of three distinctly different zones (irregular cells with a small amount of matrix in the epiphyses; flattened cells arranged in concentric ellipsoid curves; hypertrophied cells in the diaphysis) are normal in the stages preceding ossification. The formation of the periost, of the first perichondrial cylinder of bone around the cartilaginous shaft, of osseous trabeculae, the erosion of diaphysial cartilage, etc., follow in all details the normal

histogenesis of these elements, as described by Fell (1925) (see Figs. 8–12). Deviations from the normal course are: hypertrophy of bone in the concavities of abnormally curved elements (Fig. 12) and certain abnormalities in those joints which are not separated (see below).

We conclude that histological differentiation of cartilage and the first phases of ossification are independent of nerve supply and of functional activity.

THE FORMATION OF JOINTS

Two different aspects must be considered in a study of joint formation in nerveless limbs: the sculpture of the articulating surfaces of the epiphyses and the histogenesis of the joint-forming area, including its segregation or fusion. Our material is not favorable for a study of the former, since in specimens stained with methylene blue the epiphyses are colored only slightly. A fine, distinct line between two adjacent elements, however, indicates the separation which takes place in most joints on the ninth day of incubation. The heads of femora and of humeri are normal in many cases, but in several others they are abnormal and fused with their girdles (Figs. 1*b*, 4, 7*b*). Condyles and other surface elevations are usually smoother than normal. Flatness of protuberances and shallowness of depressions is a general feature, particularly in the long elements (Figs. 2 and 3). The surface details of phalanges seem to be normal in most cases.

Fusions between adjacent elements are found frequently. In nerveless limbs some joints are usually fused, whereas others are separate; no general rule for their occurrence is discovered. Fusions of all joints are found in only two cases of highly malformed transplants. The joints between long elements seem to be more frequently affected than those between phalanges. Fusions are never found in cases which are known to be innervated.

Adjacent elements usually are not fused along the entire articulating surfaces but at one corner only. This obviously results from the posture in which a nonmotile limb develops. All nerveless and poorly innervated limb transplants, which are paralyzed throughout morphogenesis, develop in a characteristic posture which may be called "static posture." In the case of the wing this corresponds to the posture assumed at rest (Fig. 5). In the leg we find a partial flexure of the metatarsus against the tibia and fibula and a slight flexure of the phalanges (Figs. 4, 8). The "static posture" is apparently the result of differential growth of the skeletal, and possibly also of the muscle, primordia.

In such legs femur and tibia are in close approximation at the innermost point of the knee joint, whereas they are quite separate at the outermost point, near the patella. The inner points are consequently preferential areas of fusion (Fig. 9). Likewise, the condyles will be expected to be better developed at the outer aspect than at the inner angle. This is, indeed, the case. The same holds for the articulation of the humerus with the radius and ulna. Similar differences at different sides of a joint are not found in articulations of metatarsals with phalanges and between phalanges as they are lined up in a slightly bent line.

A few typical cases may illustrate our findings:

Tre 634 (Figs. 1 and 2): A right leg developed inside of the coelom (illustrated in Hamburger, 1939, Fig. 12). It is innervated by two small branches of nerves 16 and 17. The transplant (at operation) was a small bud (stage 4, 64 hours) and was fixed at 18 days of incubation. The femur is fused with the pelvic girdle, but all other joints are separate, and all other articulating surfaces

are approximately normal in their gross structures. The relative smallness of the condyles at the distal end of the tibia and the shallowness of the intercondyloid fossa illustrates well the deficiencies usually found in nonfunctional limbs (Fig. 2).

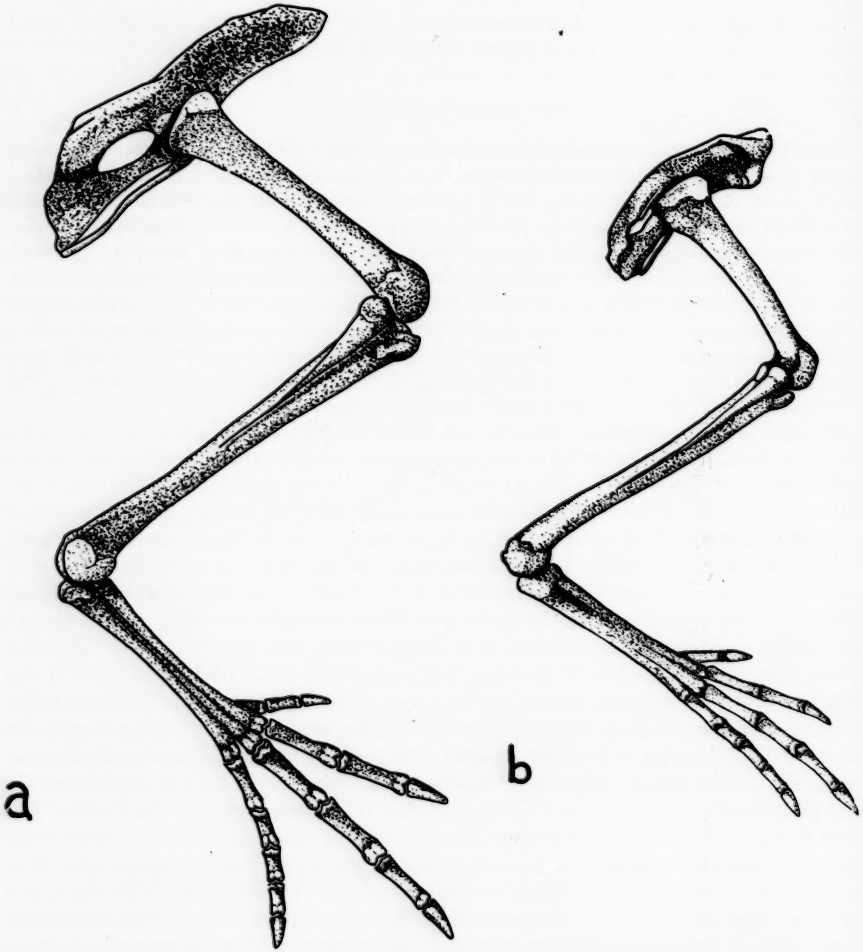

FIG. 1.—(*a*) *Tre 634:* right leg of host embryo; fixation at 18 days of incubation; dissection; 2.3×. (*b*) Same: transplant (right leg) developed inside of the coelomic cavity; poorly innervated; dissection; 2.3×.

Tre 629 (Fig. 3*b*): A right wing developed on the umbilical cord and was definitely nerveless (illustrated in Hamburger, 1939, Fig. 10). The transplant (at operation) was a very small bud (stage 3, 68 hours) and was fixed at 19 days of incubation. All skeletal elements are normal.

The head of the humerus is abnormally small, not quite typical in shape, and fused with the rudimentary pectoral girdle. All long elements are likewise fused. No articular condyles are developed at the distal end of the humerus (Fig. 3*b*). The phalanges are present and separate; their articulating surfaces are normal.

Tre 623 (Fig. 4): A left hind limb developed inside of the coelom and was attached to the inside of the body wall by a narrow stalk. It was probably nerveless. The transplant (at operation) was a very small bud (stage 3, 68 hours) and was fixed at 17 days of incubation. The first toe is missing. The girdle and all long elements are fused, and all articulating surfaces are poorly sculptured or absent. All phalanges with the exception of one pair are separate.

Tre 597 (Fig. 5): A right wing developed on the umbilical cord and was definitely nerveless (illustrated in Hamburger, 1938, Fig. 4). The transplant (at operation) was a thickening (stage 1, 52 hours) and was fixed at 9 days of incubation. All elements are normal and only slightly fused or separate.

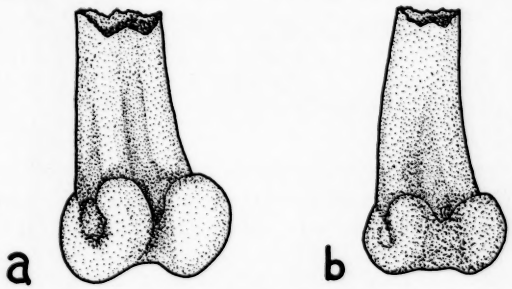

FIG. 2.—Same specimen as Fig. 1. (*a*) The condyles at the distal end of the tibia of the right host leg; 7×. (*b*) The condyles at the distal end of the tibia of the transplant; 7×.

Tre 367 (Fig. 7*b*): A right wing developed on the right flank near the midventral line; it was very poorly or not at all innervated. The transplant (at operation) was a small bud (stage 4) and was fixed at 14 days of incubation. A fusion is found between humerus and radius and ulna; all other joints are separate, and their articulating surfaces are normal. In particular, the head of the humerus which is much too large for its glenoid cavity shows a sculpture typical for its stage of development.

Tre 371 (Fig. 6): A right hind limb developed in the flank in front of the right host leg and was probably functionally innervated. The transplant (at operation) was a small bud (stage 4) and was fixed at 13 days of incubation. The pelvic girdles of host and transplant are fused. The transplant is perfectly normal in every respect.

A study of sections and of specimens stained with methylene blue shows that two different types of fusion must be distinguished, one in which the epiphyses are histologically normal and clearly demarcated but united by a small area of fibrous connective tissue and another in which adjacent elements are connected by cartilaginous tissue which has obscured the border line between the two elements. This latter mode of fusion is rare, whereas the former type is frequent. In specimens stained in methylene blue fusion by cartilage is indicated by continuity of the deep blue stain (e.g., Fig. 4).

The two types may be readily explained on the basis of our knowledge of normal joint formation. The histogenesis of the avian knee joint is described by Fell and Canti (1934). The knee-joint region consists of a dense mass of undifferentiated "mesoderm" at a stage

when femur, tibia, and fibula begin to chondrify at their diaphyses. The direct continuity between the three elements persists for some time, and in a 5-day embryo "in thionin-stained preparations it is interesting to note that cartilage matrix of a very young and

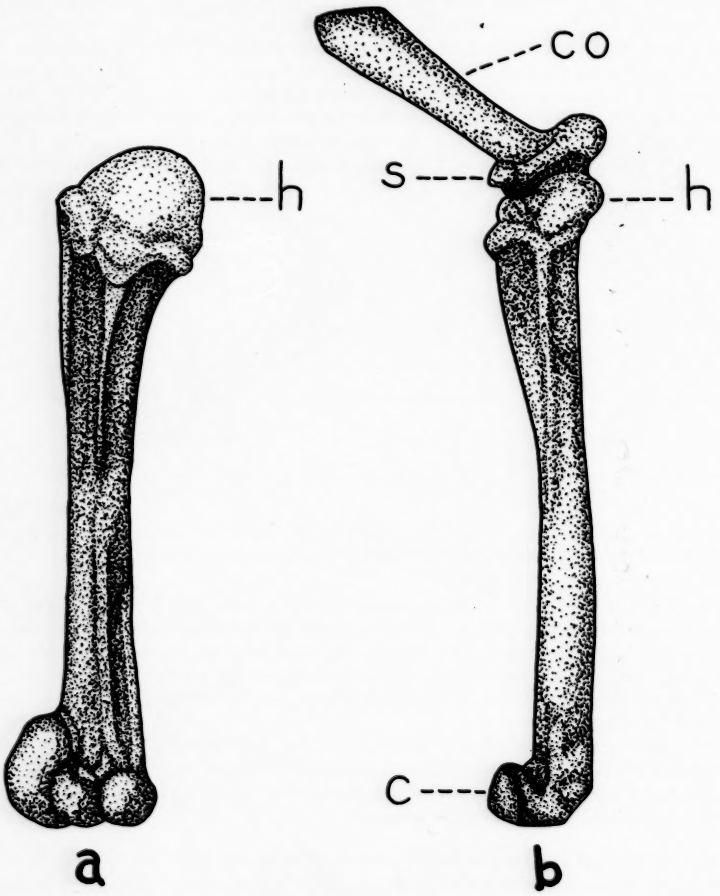

FIG. 3.—(*a*) *Tre 629:* right humerus of host; fixation at 19 days of incubation; dissection; 10×. (*b*) Same: humerus and rudimentary pectoral girdle of transplant (right wing) developed on the umbilical cord; nerveless; dissection; 10×; the humerus was fused with the radius and ulna and was severed at the level of the joint at *c; co*=coracoid; *h*=head of humerus; *s*=scapula.

immature type extends right across the line of the joint . . . . " (Fell and Canti, 1934, p. 320). Cartilage matrix is still visible in the joint area of the 6-day embryo, but about the eighth day it is replaced gradually by, or transformed into, loose fibrous tissue in

which a cleft appears. The loose tissue disappears eventually. According to our observations, joint formation is essentially similar in the other joints.

The first, more frequent, type of fusion (by connective tissue) is explained readily by assuming that joint formation proceeds normally but stops short of the final step—the segregation of adjoining elements. This type we may consider as a "primary fusion," or "nonfission" (Warren, 1934). In the second type of fusion (by cartilage) we assume either that the normally transitory phase of cartilaginous differentiation in the joint persists instead of undergoing regressive changes or that fibrous joint tissue is formed first but replaced later by cartilage in a second atypical phase of chondrification. This latter interpretation which must be designated as a "secondary fusion" by cartilage is probably correct. Fell and Canti (1934) found in tissue cultures of knee-joint regions exactly this sequence of events—the formation of a marked articular line of dense connective tissue by the fourth day of cultivation, i.e., at a total age of 8 days.

> After 6 days cultivation *in vitro*, further joint differentiation ceases and actual fusion of the bone rudiments takes place. This is shown in the living culture by the fact that the dark line across the knee joint becomes less and less distinct. . . . . Sections of an explant cultivated for 17 days show direct continuity between the three cartilages at their points of contact [*op. cit.*, p. 324].

This the authors call "secondary fusion." The fact that in our material primary fusions are frequent and secondary fusions rare supports further this interpretation. Occasionally the same joint shows both types of fusion. One knee joint (Fig. 8) is found to be separate at the outer aspect, i.e., in the region of the patella. Primary fusion of femur and tibia by fibrous tissue is found in the middle region (Fig. 9) and secondary fusion by cartilage in the inner angle (Fig. 10).

Such observations suggest that growth pressure or other mechanical conditions at the point where opposed elements meet each other are responsible for secondary cartilage formation. Glücksmann (1939) demonstrated the transformation of fibrous tissue into cartilage under similar conditions of slight pressure in tissue-culture experiments. No sectioned cases older than 12 days are available. It will be of interest to find out if a secondary cartilage fusion is more frequent in later stages than in younger, i.e., whether the process of secondary fusion is progressive during development.

DISCUSSION

The present material demonstrates to what a remarkable degree of perfection the differentiation of the limb skeleton of the chick may proceed in the absence of innervation and of function. The method of intra-embryonic transplantation provides for optimal conditions and, therefore, reveals the inherent potencies of the transplants to their fullest extent. In previous isolation experiments of limb primordia of about the same stages (48–72 hours of incubation) in which the method of chorio-allantoic grafting was used (Murray and Huxley, 1925; Murray, 1926, 1928; Hunt, 1932) abnormal skeletons were obtained, in which many elements were distorted or missing and many were fused. It is now obvious that these deficiencies, with the exception of the fusions, are due not to a lack of self-differentiating potencies of the transplants but to spatial restrictions and other unfavorable developmental conditions on the chorio-allantoic membrane. The failure of 2-day primordia to differentiate into any structures resembling a normal skeleton

(Murray, 1928) shows merely that these young stages are more susceptible to the unfavorable conditions of the membrane than are older stages.

Functional activity and joint formation.—Even the best-developed cases of our nerveless or poorly innervated transplants show more or less severe defects in joint formation. Primary and secondary fusions (see p. 374) are common, particularly in the joints of girdles and between long bones. Other joints, particularly those between phalanges, may be completely separate. The fact that by no means all joints of a nerveless limb are necessarily fused indicates that the embryonic movements are at best a subsidiary factor in bringing about separation. At present we have no explanation for the observation that some joints are fused while others are not. Moreover, it was pointed out heretofore (p. 377) that movements can be instrumental only in bringing about the final step in joint formation—the formation of a cleft; whereas all preceding steps (differentiation of the articular cartilages, of fibrous articular tissue between the articular cartilages, etc.) proceed normally, not only in our transplants but even *in vitro* (Fell and Canti, 1934). In addition to fusions, deficiencies in the sculptural details of articulating surfaces are common; condyles are smoother, and grooves are shallower than normal.

These observations are in full agreement with earlier experiments on nerveless limbs in anurans and in the chick. Of the crucial experiments we mention those of Braus (1909, 1910), who transplanted fore-limb buds of *Bombinator* heterotopically and found that the girdles were abnormally small and the limbs immobilized. The joint separation was complete, but the head of the humerus, which did not fit into the glenoid cavity, showed deficiencies in its surface structure. In our own experiments on nerveless limbs of frogs (Hamburger, 1928) it was observed that a separation of joints was usual, but fusions occurred occasionally, and minor details of the surface sculpture were deficient. For the chick we mention the extensive chorio-allantoic grafting experiments of Murray (1926, 1928), whose best-developed cases proceed far toward normality. Finally, the tissue-culture experiments of Fell and Canti (1934) showed that the presumptive knee-joint area of a leg bud of a 4-day embryo when isolated proceeded to almost complete joint formation but stopped short of the final step, the separation of femur and tibia. Murray and Huxley (1925) and Murray and Selby (1930) carried the analysis a step further by showing that an articulating surface will self-differentiate nearly to perfection even in the absence of the contiguous element. Isolated primordia of humeri and femora were reared on the chorio-allantoic membrane. All typical parts (head, condyles, etc.) were formed, but the same imperfections of surface details were observed as in our experiments. Fusions of contiguous elements were also observed by Warren (1934) in experiments, in which halves of wing buds were allowed to differentiate *in situ*, and by Schmalhausen (1925) and by Brunst (1927, 1932) in regenerating limbs of salamanders which were paralyzed by nerve resection.

The role which innervation plays in joint formation is a minor one; yet it is desirable to analyze the mechanism by which it enters into this complex process. It may be operative in one of the following ways. (1) Lack of innervation results in a complete paralysis of the limb throughout development. The occasional nonfission of adjacent elements may be ascribed to this immobilization. Slow gliding movements are possibly a subsidiary factor in bringing about the separation which is thus doubly assured. (2) The deficiencies in sculptural details we are inclined to explain in a different way. The absence of innervation results in an atrophy and degeneration of the skeletal musculature, which is much more radical in the chick than in the frog (Hamburger, 1928). It is likely that the tendi-

nous attachments of such muscles are structurally deficient, and it is certain that no pull is ever exerted on these points of attachment. It is conceivable that the formation of protuberances, i.e., local growth of cartilage or local bone deposition, requires normal and functionally active attachments of tendons and ligaments, i.e., the exertion of pull and stress. The importance of such factors in the mechanism of chondrogenesis is clearly brought out by Glücksmann (1939). (3) The possibility that these abnormalities may be caused by disturbances in the metabolism of the skeletogenous tissue should not be lost sight of. The nervous system may exert an indirect trophic control on skeletogenesis. It is our experience as well as that of other investigators that skeletons which are otherwise highly abnormal show an increased tendency toward fusions. Fell and Canti (1934) and Fell and Landauer (1935) observed that fusions of tibia and fibula were frequent *in vitro* and more marked if a special growth-restricting medium were used. Schmalhausen (1925) found fusions of cartilages in the limbs of salamanders which had been starved or exposed to high temperatures during morphogenesis. The experimental evidence available does not permit us to decide between these three alternatives. We wish to point out, however, that from the point of view of developmental mechanics "functional activity" covers a complex of heterogeneous factors in skeletogenesis.

Whereas the primary development of the limb skeleton is thus shown to be self-differentiation to a high degree, extrinsic factors become of increasing importance in later phases of bone development. It is well known that the details of articulating surfaces, and, in particular, the "trajectorial" arrangement of the trabecles—are formed under the influence of external mechanical conditions to which they are exposed (tensions, stresses, etc.) and that structural transformations are brought about easily as a response to changed mechanical factors, e.g., in fractured bones. These findings underscore Harrison's warning (1933) against the indiscriminate acceptance of the concept that differentiation proceeds universally from an undetermined state to one of rigid mosaic development.

SUMMARY

Nerveless and poorly innervated wings and legs of chick embryos are obtained by transplantation of early primordia to a ventral position, into the coelomic cavity, or to the umbilical cord. Investigations of the skeleton of the transplants, 8–18 days of age, in whole-mount preparations and in sections gives the following results:

1. Morphogenesis in the absence of innervation is normal in a high percentage of cases. Hypodactyly and hypophalangy are the most common abnormalities in transplants.

2. Chondrification and the first phases of ossification proceed normally in the absence of innervation.

3. Joint formation in nerveless limbs may be almost perfect and complete. Smoothness of articulating surfaces and fusions of joints, however, are found in the majority of cases. Two types of fusion, primary and secondary, are distinguished, and their origin is discussed.

4. Skeletal growth of transplants is reduced approximately 20 per cent. A possible trophic effect of the nervous system is discussed.

5. The girdles are more poorly developed than are the free limbs. Particularly the dorsal girdle elements, the scapula, and the ilium, are disproportionately small. This result makes it likely that the primordia of scapula and ilium are located in part outside of the transplanted primordium.

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PLATE I

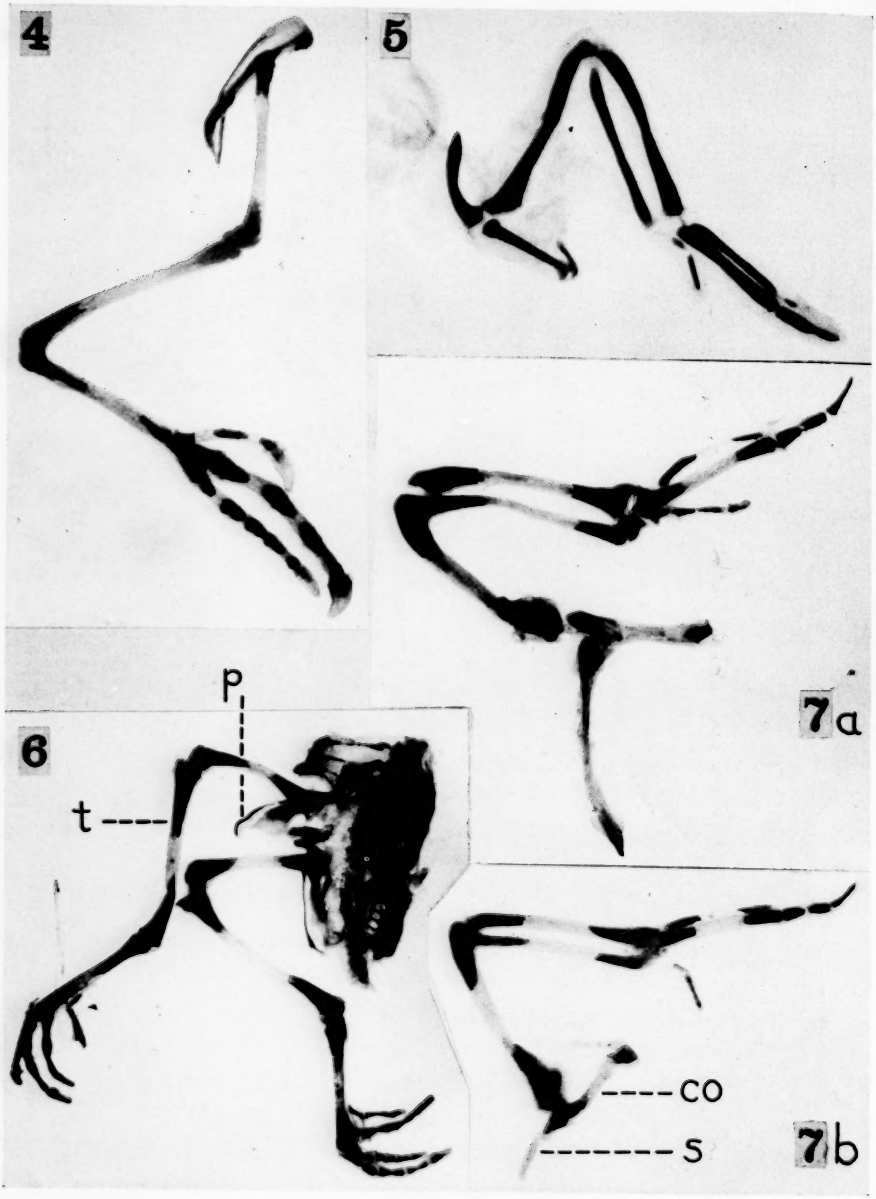


PLATE I

FIG. 4.—*Tre 623:* Transplant (left leg) developed inside of coelomic cavity, nerveless; fixation at 17 days of incubation; methylene blue; 3×.

FIG. 5.—*Tre 597:* Transplant (right wing) developed on the umbilical cord; nerveless; fixation at 9 days of incubation; methylene blue; 7.5×.

FIG. 6.—*Tre 371:* Transplant (right leg) developed on right flank; probably innervated; ventral view; fixation at 13 days of incubation; methylene blue; 2.5×; *p*=pubis of transplant; *t*=transplant.

FIG. 7*a*.—*Tre 367:* Right host wing and pectoral girdle, disarticulated; fixation at 14 days of incubation; methylene blue; 4.3×.

FIG. 7*b*.—Same: Transplant (right wing) developed on right flank near ventral midline; probably poorly or not innervated; methylene blue; 4.3×; *co*=coracoid; *s*=scapula.

PLATE II

FIG. 8.—*Tre 615:* Transplant (right leg) developed on the umbilical cord; nerveless; fixation at 12 days of incubation; Heidenhain's hematoxylin; 7×; *k*=knee joint.

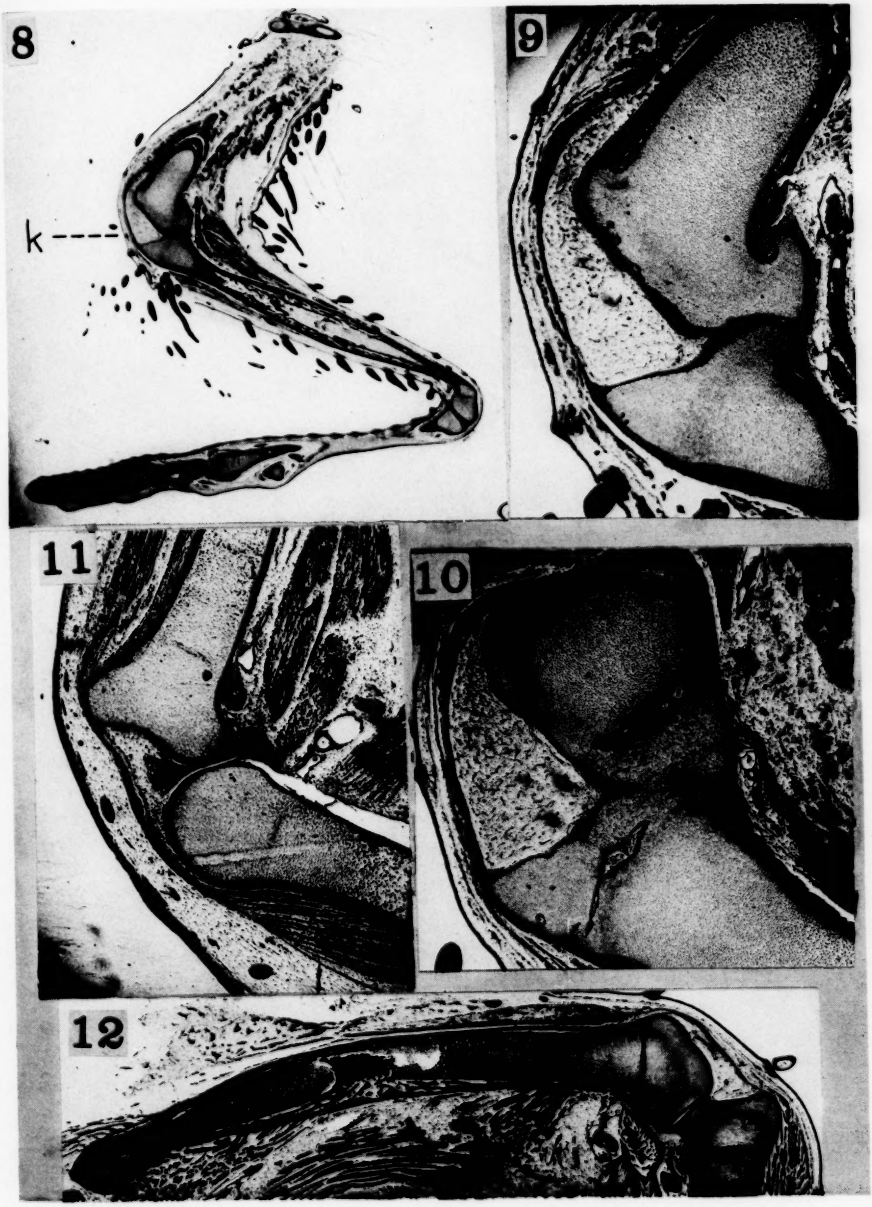
FIG. 9.—Same: Knee joint; primary fusion (see text); 27×.

FIG. 10.—Same: Knee joint; secondary (cartilage) fusion; section median to Fig. 9; 27×.

FIG. 11.—Knee joint of normal 9-day embryo; Heidenhain's hematoxylin; 27×.

FIG. 12.—*Tre 572:* Transplant (right leg) developed in coelomic cavity; nerveless; longitudinal section through femur and knee joint; fixation at 10 days of incubation; Heidenhain's hematoxylin; 16×.

PLATE II



THE STIMULUS FOR MATURATION AND FOR OVULATION OF THE FROG'S EGG

(One plate)

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IT IS well known that many Amphibia will ovulate in response to injections of anterior lobe pituitary gland from the same species. Such ovulation is normal, and during release and passage of the egg through the oviduct maturation changes occur leading to formation of the first polar body and the second maturation spindle, after which fertilization is possible. In *Rana* ovulation can be produced by pituitary from most Anura and sometimes by large amounts of *Triturus* pituitary, mammalian pituitary extracts, and human pregnancy urine (see review by Creaser and Gorbman, 1939). *Xenopus laevis* responds consistently to large doses of mammalian pituitary and human pregnancy urine, also to progesterone and several other steroids (Bellerby, 1933; Zwarenstein, 1937; Shapiro, 1936). The injected pituitary appears to be able to act directly upon the ovary, for ovulation can be induced after removal of the host pituitary (although under such circumstances there may be quantitative alterations in the reaction) (Novelli, 1932; Canturisvili, 1936; Wunder, 1938*b;* Rugh, 1939) or of many other organs, including the thryoid and adrenals (Novelli), and also after destruction of the central nervous system by "pithing." Heilbrunn, Daugherty, and Wilbur (1939) found that excised frog ovaries will ovulate in suspensions of frog pituitary but not in plain Ringer's or in muscle-tissue suspensions (one experiment). Also Zwarenstein (1937) obtained ovulation in excised ovaries of *Xenopus* with progesterone suspensions.

Thus there is no evidence to suggest the involvement of any organ other than the pituitary and ovary in ovulation of the frog's egg, but some suggestion that a specific pituitary stimulus is not required. Evidence regarding maturation of the egg is less clear. Heilbrunn, Daugherty, and Wilbur report maturation of eggs shed by excised ovaries, but their data concern nuclear breakdown, which is not a very demanding criterion. Likewise, their photographs are not convincing. Zwarenstein found that eggs released by progesterone from normal animals were fertilizable, but no data regarding eggs shed by hypophysectomized animals or excised ovaries are given.

PROBLEM

Since the excised ovary affords a better opportunity than does the intact animal for controlled experimentation, with certainty as to the direct application of stimuli to the ovary itself, this method would appear to be highly favorable for investigation of the mechanisms involved in maturation and ovulation of the egg. However, it is essential to be sure that the changes induced *in vitro* are identical with those occurring *in vivo* under pituitary stimulation and in spontaneous ovulation. The experiments here reported were performed to determine whether this is so and also to determine whether both ovulation and maturation are initiated by a specific substance confined, in *Rana*, to the pituitary gland.

PROCEDURE

The experimental procedure used was essentially that of Heilbrunn, Daugherty, and Wilbur. All the animals were *Rana pipiens brachycephala* Cope (Stejneger and Barbour, 1939)] from upper Vermont and the St. Lawrence Valley. Most of the experiments were on animals taken from hibernation in April, but a second series of experiments was carried out in November. Fragments of ovary, each containing about 100 eggs, were first rinsed in Ringer's and then immersed in a suspension made by mashing either one anterior lobe pituitary or about an equal volume of another tissue in 30 cc. of Ringer's. Other control fragments were kept in plain Ringer's. In each experiment parts of a single ovary were used in parallel pituitary and control preparations. In some cases whole ovaries were used with 2 pituitaries in 30 cc. of Ringer's, the other ovary being treated with an equal or larger amount of another tissue. All experiments were conducted at about 22° C. At intervals of about 6 hours ovulated eggs were counted and fixed in Smith's fluid. The fragment of ovary remaining after ovulation had stopped was preserved in the same way.

OVULATION

Table 1 summarizes the results of the spring experiments. A significant fact emerging is that a number of tissues other than pituitary caused some ovulation. Clearly, pituitary is the most potent and the only one which gave consistent results, but ovulation

TABLE 1

OVULATION *in vitro*: SPRING ANIMALS

Tissue	Number of Experiments	Number of Positive Results	Mean Percentage of Eggs Shed in Positive Cases (Approx.)	Tissue	Number of Experiments	Number of Positive Results	Mean Percentage of Eggs Shed in Positive Cases (Approx.)
Pituitary	10	10	12	Stomach	4	0	0
Pituitary*	8	8	8	Blood	3	0	0
Muscle	7	1	15	Liver	2	0	0
Medulla*	5	2	2	Heart	2	0	0
Cerebrum	2	0	0	Pancreas	2	0	0
Cerebrum*	2	1	2	Intestine	2	0	0
Adrenal	4	2	2	Ovary	1	0	0
Kidney	2	1	3	Ringer's	5	0	0
Testis	2	1	2				

* Whole ovaries; more control tissue than pituitary.

was obtained with 6 out of 13 other tissues tried. The number of experiments was too small to exclude the possibility that other tissues might sometimes be effective. That ovulation was due to the tissue present and not to spontaneous ovulation of the excised ovaries is shown by the following facts: (*a*) about 100 females from the same batch of animals kept in the laboratory failed to ovulate spontaneously; (*b*) control fragments of the same ovaries kept in plain Ringer's did not ovulate; (*c*) unattached fragments of ovary, when left in the body cavity, do not shed their eggs. Three experiments performed with pituitary on ovary fragments in early October were negative, but at this season even

intact animals often fail to respond to pituitary injections. Six pituitary experiments performed during November were all positive. Since the ovary is less sensitive to the ovulation-inducing substance of the pituitary in November than in April (Wunder, 1938*a*) it is not surprising that muscle and medulla failed to induce ovulation in the autumn.

In 4 November experiments the concentration of pituitary was varied. For each experiment a suspension of female pituitaries was prepared and repeatedly diluted to give preparations containing from 4 pituitaries to 1/128 of a pituitary in 10 cc. of Ringer's. A single ovary was divided into pieces containing 5–10 eggs; and about 125 eggs, taken at random, were put into each preparation. Possible regional variations in the sensitivity of the ovary were thus eliminated so far as possible. Dilutions containing $\frac{1}{64}$ or less of a pituitary in 10 cc. failed to cause ovulation; the results with greater concentrations

TABLE 2

CONCENTRATION AND OVULATION

Experiment		Amount of Female Pituitary in 10 Cc.							
		4	2	1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$
I	Eggs used	.....	226	204	197	179	192	172	.....
	Percentage shed	.....	22.6	10.8	15.2	11.7	1.6	3.5	.....
II	Eggs used	122	103	113	85	104	110	.....	.....
	Percentage shed	13.1	23.3	32.7	18.8	18.3	32.7	.....	.....
III	Eggs used	.....	94	87	98	74	99	86	88
	Percentage shed	.....	22.4	26.4	55.2	68.9	43.5	59.3	5.8
IV	Eggs used	.....	150	154	135	131	148	141	124
	Percentage shed	.....	19.6	55.8	34.8	16.8	30.4	29.8	3.2
Total eggs used		122	573	558	515	488	549	399	212
Mean percentage shed		13.1	21.8	30.1	28.5	23.1	23.1	24.8	4.3

are summarized in Table 2. It will be seen that there is a decrease in response at both high and low concentrations. The mean optimum concentration was about one pituitary in 10 cc., but the optimum was perhaps different in each experiment. When a statistical test for effectiveness of all the active concentrations was made, a significant variation arising from the concentration differences was found ($\chi^2 = 56.2$; $n = 6$; $P < 0.01$). When classes 2 and $\frac{1}{4}$ were contrasted with the optimum classes 1 and $\frac{1}{2}$ by means of a fourfold table, ovulation was found to be significantly less in the former ($\chi^2 = 7.84$; $n = 1$; $P < 0.01$). Thus, for November frogs under these experimental conditions, there is an optimum concentration, represented by $\frac{1}{2}$–1 pituitary in 10 cc. of Ringer's, above and below which the ovulation response *in vitro* is reduced. *In vivo* experiments have not shown that ovulation decreases when an excess of pituitary is injected. However, it must be noted that this effect is not seen *in vitro* until sixty-four times the minimum effective amount is used, i.e., an amount corresponding to about 128 pituitaries for an intact animal.

The amount of ovulation obtained was always considerably less than that ordinarily

attained *in vivo* (the maximum response was 68.9 per cent,[1] and the mean for November and spring experiments was 23.5 per cent and 12 per cent, respectively). This was apparently due to disintegrative changes in the ovarian fragments. The first eggs to be released usually fell from the follicles about 10 minutes after their initial rupture. But extrusion became progressively slower, and when eggs had ceased to fall from the ovaries there were always to be found follicles which had ruptured normally but had failed to extrude their eggs. Ovaries stimulated *in vivo* and excised during ovulation similarly fail to shed all their eggs.

NORMALITY

That the process of ovulation observed in excised ovaries is (until impeded by cytolysis of the ovary tissue) essentially identical with that occurring *in vivo* is indicated by the following similarities between them: (1) The duration of the latent period between initial stimulation and initial follicular rupture is similar since ovulation began after 14–19 hours' immersion. (2) Increasing the amount of pituitary used does not noticeably affect the length of this latent period. The number of eggs shed is roughly proportional to the amount of pituitary used in both cases (at least when near the minimum threshold concentration of pituitary). (3) Comparison of ovulation in excised ovaries with that in ovaries stimulated *in vivo* (described by Rugh, 1935) failed to show any difference in the visible characteristics of the process in the two cases. (4) Shedding of eggs invariably took place through the normal point of dehiscence, i.e., to the exterior through the area of attachment of the follicle to the ovary wall. In two experiments a single lobe of an ovary was tied off and pituitary suspension was injected into the cavity of the isolated lobe. The whole ovary was then immersed in 30 cc. of Ringer's. Most of the eggs in the injected lobes were shed and, without exception, to the exterior. No eggs were released from other parts of the ovaries. (5) Maturation of the egg occurs during muscle- and pituitary-induced shedding of eggs from excised ovaries as in normal ovulation (see below).

MATURATION

That maturation changes, essentially identical with those occurring in the intact animal, take place in eggs released from excised ovaries by pituitary has been established by four methods: (1) Three batches of pituitary-released eggs were examined for polar bodies, which were found in all 180 eggs. (2) Fifty eggs released from an isolated lobe of an ovary injected with pituitary were dissected under a binocular microscope, but in no case was an intact germinal vesicle found. Fifty eggs remaining in the uninjected parts of the same ovary all had intact germinal vesicles. (3) Eggs taken from the coelom of an ovulating female, and consequently without oviducal jelly coats, cannot be fertilized. In order to test the fertilizability of eggs from excised ovaries it was thus necessary to provide them with normal jelly coats. About 150 eggs released from an excised, pituitary-treated ovary were placed, 48 hours after the initial stimulation, in the body cavity of a female ovariectomized 48 hours previously (the same animal from which the ovary used had been taken). Twenty-four hours later the eggs were taken from the "uteri" and artificially inseminated. About one-third of the eggs were already cytolyzing. The remainder fertilized and began cleavage. Most of them developed through gastrulation, and 39 hatched as swimming larvae. Since a number of the eggs

[1] Complete ovulation has since been obtained in many cases.

had already begun to cytolyze at the time of fertilization, it is not surprising that many of the remainder developed abnormally. Cytological study showed that many eggs were abnormal after 2 days in the suspensions (Fig. 5). (4) The cytological methods used proved highly favorable for the study of maturation processes. Smith's fluid fixed spindles in a light-refractive state, making them very obvious in the absence of any counterstain. The Feulgen technique stained chromosomes an intense red, while yolk granules were colored only faintly. In eggs released by pituitary and by muscle suspensions, which were fixed shortly after shedding, cytological figures were obtained showing 13 tetrads dispersed about a spindle which was oriented radially in the egg and at metaphase of the first maturation division (Fig. 1). Anaphase showed 13 dyads in the egg and 13 in the polar-body projection (Fig. 2). In 4 cases the second maturation spindle, with its associated chromosomes, was found beneath the completed first polar body (Figs. 3 and 4). This figure is identical with that found in spontaneously ovulated eggs taken from the "uterus." Eggs normally remain in this state until fertilized (Parmenter, 1933; Porter, 1939). The stages seen are the same as those observed by E. J. Ryan (unpublished, 1940) for the normal maturation of the eggs of *R. pipiens* and also resemble those described by King (1901) for the toad. Thus, it seems probable that entirely normal maturation took place in at least some of the eggs released *in vitro* by pituitary and by muscle-tissue suspensions.

In 13 out of 138 eggs examined cytologically the germinal vesicle was found to be intact even 40 hours after ovulation (Fig. 6). These eggs included all that had been released by kidney (3), testis (2), and adrenal (4), 1 out of 15 released by muscle, and 3 out of 114 released by pituitary. Eggs released by brain tissues were not examined cytologically. Seemingly true ovulation had occurred in these cases, since all eggs were free from investing follicle membranes. Thus, ovulation can apparently take place independent of maturation.

DISCUSSION

There is thus much evidence that ovulation and maturation induced *in vitro* by means of pituitary suspensions differ in no important respect from the corresponding processes occurring *in vivo*. We are therefore of the opinion that the method described here is suitable for further study of the ovulation and maturation processes in the frog.

Muscle suspensions also can stimulate both processes, at least in highly sensitive spring ovaries. Other tissues can cause ovulation but apparently not maturation. There is some suggestion that these differences may be only quantitative, since maturation did not take place in a few eggs released by pituitary and muscle. Since in *Xenopus* a variety of steroids as well as several gonadotropic preparations can induce ovulation (Shapiro, Zwarenstein), there is no reason to suppose that the ovulation in *Rana*, induced by brain, muscle, adrenal, testis, and kidney suspensions, is due to the same substance that is present in the pituitary extracts. It is evident, however, that, whatever the nature of the ovulation-inducing stimulus, it is not entirely confined in *Rana* to the pituitary gland.

The results of these experiments are consistent with the hypothesis (Rugh, 1935) that the release of the egg is due to action upon the follicular membranes of a digestive enzyme produced by the egg under pituitary stimulation, although the activator need not be an enzyme. In the ligature experiments eggs were shed from the isolated lobes, but not from the rest of the ovary. And the eggs were shed to the exterior, although

the stimulating substance was inclosed in the ovarian cavity. If rupture were due to a diffusible substance which had a differential action on the point of attachment of the follicle to the ovary, it would be difficult to explain the absence of rupture in eggs not isolated but adjacent to the ligature. It might, then, be tentatively suggested that the stimulating substance acts indirectly by means of a mechanism in the egg or follicle. In any event, ovulation could hardly be due to a general histolytic action of the suspension upon follicular tissue, especially since release of eggs ceases when histolysis becomes visible. That ovulation is not due to release of any substance from the germinal vesicle as a result of maturation is indicated by the fact that eggs may sometimes be released without breakdown of the germinal vesicle.

The quantitative methods described provide the basis for a more satisfactory method of comparatively assaying frog pituitaries for ovulation-producing potency than any hitherto available. While there are quantitative differences discernible in the reaction of intact animals to graded doses of pituitary (Rugh, 1937; Wunder, 1938*a*), there is a great deal of individual variation in the sensitivity of different animals to the same dose of pituitary. Accurate assay by methods based on the intact animal, therefore, requires use of many animals and is possible only when large amounts of pituitary are available for test. Both of these difficulties can be avoided by use of excised ovaries. A single ovary provides ample material for many tests, and regional variation in the sensitivity of the follicles can be eliminated by random selection of eggs used in parallel test and control preparations. The amount of pituitary needed for a test is very small. If the threshold concentration for response were used as a basis for assay, a satisfactory comparison of potency could probably be made between 2 single pituitaries, since 1 pituitary is (in November) enough for about 30 threshold experiments.

SUMMARY

1. Ovulation and maturation of the frog's egg can be induced in excised ovaries by means of anterior lobe pituitary suspensions and sometimes by muscle suspensions. Ovulation can sometimes be induced by suspensions of several other tissues.

2. Evidence is adduced for the contention that ovulation and maturation produced in this way are essentially identical with the same processes induced *in vivo*. The *in vitro* method is therefore considered suitable for further study of ovulation and maturation processes in the frog.

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PLATE I

FIG. 1.—Section of an egg released *in vitro* by muscle suspension. Some of the tetrads on the first maturation spindle are very evident. × 750.

FIG. 2.—Section of an egg released *in vitro* by pituitary suspension. Several dyads are visible in the first polar-lobe projection; the spindle was cut diagonally, and the dyads remaining in the egg are in adjacent sections. × 750.

FIG. 3.—Section of an egg released *in vitro* by pituitary suspension. Chromosomes are seen in the indistinct first polar body. The second maturation spindle is in metaphase, and the chromosomes seem small because they are viewed on end. × 750.

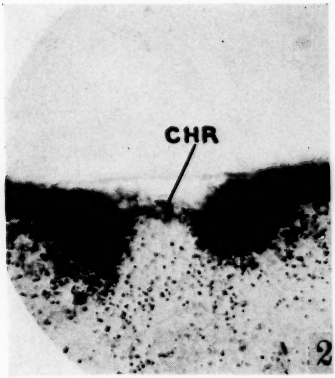
FIG. 4.—Section of an egg released *in vitro* by pituitary suspension. The first polar body is distinct under the egg membrane, and the second maturation spindle is in an adjacent section. × 750.

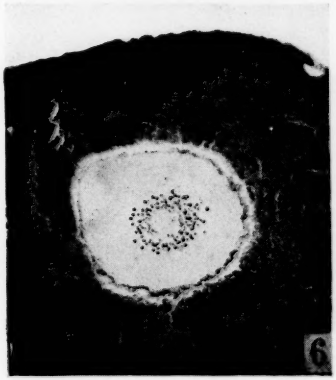
FIG. 5.—Section of an egg released *in vitro* by pituitary suspension and fixed 48 hours later. The chromatin is clumped at the periphery, and a spindle is not visible. × 750.

FIG. 6.—Section of an egg released *in vitro* by pituitary suspension. The germinal vesicle is still intact; note the absence of follicle cells. × 60.

These eggs were fixed in Smith's fluid and stained by the Feulgen method. Although the chromosomes and pigment granules appear black in the photographs, it must be remembered that in the sections the chromosomes were bright red.

PLATE I





PHYSIOLOGICAL CHARACTERISTICS OF THE DIAPAUSE GRASSHOPPER EGG. I. THE STABILITY OF THE DIAPAUSE CONDITION[1]

(Three figures)

JOSEPH HALL BODINE AND WILBUR A. ROBBIE
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THE mechanism of diapause in the Orthoptera is poorly understood. Bodine (1932) has postulated the existence of a diapause factor *x*, responsible for the appearance of the resting condition in the egg of the grasshopper, *Melanoplus differentialis*. This developmental and physiological block may be prevented or broken by prolonged exposure to low temperature (Bodine, 1925 and later; Slifer, 1932; Burdick, 1937). Its variation in potency within the eggs of a given pod is shown by the wide range of hatching time for individuals when kept at a constant high temperature (Slifer, 1931; Burdick, 1937).

Detection of changes in the egg of the grasshopper during the diapause period has received little attention. Slifer (1931) states that mitotic division stops rather abruptly within a week after the onset of diapause and the embryonic cells remain in the resting condition until postdiapause development starts. Bodine (1929) showed that the weight of the egg increased only slightly, if at all, from the start of diapause to the one-hundredth day. The mean level of respiration of eggs from the thirtieth to the one hundred and seventy-fifth day remains low (Boell, 1935; Robbie, Boell, and Bodine, 1938). It is the aim of the present investigation to study certain conditions which exist in the egg during the diapause period, in the hope that a better understanding of the regulatory mechanism may result.

The morphological development which follows the breaking of diapause is easily observed. By treating eggs of different ages with agents that tend to break diapause, it is possible to determine the relative potency of the block by noting the number of individuals which are induced to resume development. Experiments of this type are reported in this publication; studies on changes in density, weight, and other characters during diapause will be reported in a further paper.

MATERIAL AND METHODS

Eggs of the grasshopper, *M. differentialis*, were used in all the experiments. Within 24 hours of laying, the pods were collected and placed at 25° C. Particular care was taken to insure satisfactory conditions of aeration and moisture during storage. The entire pods were kept in covered dishes on a firm layer of damp, but not wet, sand, and at intervals of a few days the covers were removed and the pods agitated.

By observing the position of the embryo's pigmented eyes within the egg, it is possible to detect postdiapause development without in any way disturbing the protective mem-

[1] Aided by a grant from the Rockefeller Foundation for work in cellular biology.

branes. To do this more readily, light from a microscope lamp was reflected from a mirror below the stage of a dissecting microscope in such a way that the rays passed obliquely through the eggs (Fig. 1). When observed in this fashion, the pigmented eyes are in conspicuous contrast to the lighter, remaining portions of the embryo and yolk. When the light comes from above, the eggs appear opaque, and it is difficult to see the eyes.

A Petri dish with three paraffin chambers and a narrow connecting channel the width of the low-power dissecting microscope field (Fig. 1) is a help in sorting eggs. When in use, the dish is filled with water, and the eggs are placed in one chamber. As each one is pushed through the field of view in the channel, it is examined and directed to either of the other sections—the satisfactory individuals to one, the discards to another.

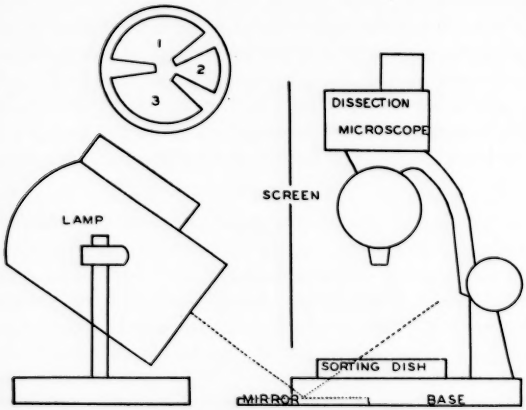

FIG. 1.—Diagram of setup used in examining eggs. Inset shows top view of sorting dish with its three compartments for unsorted eggs, discards, and satisfactory individuals.

The eyes during the diapause period and for the first 3 days of postdiapause development lie near the micropyle end of the egg. On the seventh day at 25° C., however, they are considerably increased in size and have moved to the anterior end (Slifer, 1932). It is thus possible by examination to select for use eggs which contain embryos and to note if growth occurs after treatment. The effects of experimental conditions on the breaking of diapause were made by observing development in this manner.

Eggs used in the experiments reported here, after being removed from the pods, washed, and the obviously bad ones discarded, were counted into lots of 100 and stored in castor dishes on damp filter paper. Each group was examined under the binocular dissecting microscope to be certain that every egg contained an embryo. All the dishes were kept continuously at 25° C. except those of the three experiments in which the effects of other temperatures were studied. At 7 days from the start of each determination the control eggs were examined to see how many had begun to develop. The experimental lots were counted similarly a week after the end of the treatment period. To observe the effect of centrifugal force, eggs were centrifuged in glass tubes under water for 20 minutes, at top speed in a clinical centrifuge (660 × gravity). Exposures to tem-

peratures of 0°, 10°, and 36° C. were carried out in refrigerators and in an electric oven, respectively. In the oxygen, nitrogen, and carbon dioxide experiments a sealed desiccator vessel containing dishes of eggs was filled with the gas. Eggs kept under water remained in their castor dishes with ½ cm. of liquid covering them. Mild dehydration was effected by leaving eggs on dry filter paper. For more intense drying action they were kept on paper saturated with a ten-times isotonic sodium chloride solution. Treatment by all these methods (except centrifuging) was for a 6-day period, and at the close of this time the eggs were returned to the same conditions as the control groups.

RESULTS AND DISCUSSION

It is evident from the results presented in Figure 2 that there is a definite age difference in regard to susceptibility to the breaking of diapause. Those eggs that have just

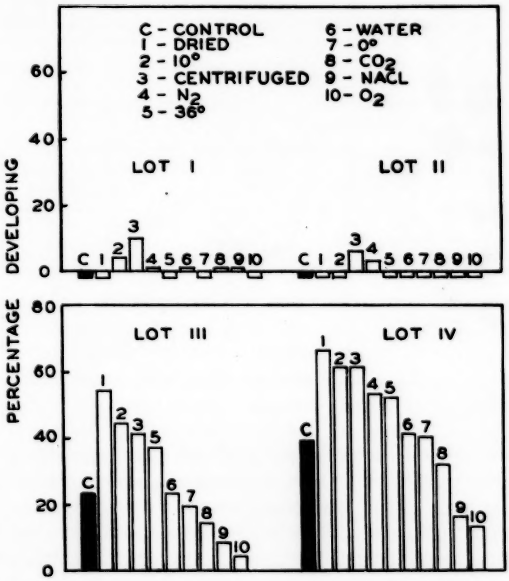

FIG. 2.—Graphs showing the effects of various treatments on breaking the diapause block in grass hopper eggs (for details see text). Ordinates represent the percentage developing within 7 days after end of treatment. At the start of the experiment the ages of lots 1–4, respectively, were 21, 39, 153, and 158 days; areas below the abscissa in lots 1 and 2 represent zero effect.

entered diapause and the ones that have been in this resting period for less than 3 weeks are almost entirely unaffected by any of the experimental treatments. The two lots that have been in diapause for about 20 weeks show much less ability to maintain themselves in this stage, and controls as well as experimentals have a greater tendency to develop. That this age effect is consistent and is not due to a peculiarity in choosing the samples is shown in Table 1, which represents results of centrifugation studies on 73 different lots of eggs with ages varying from 20 to 200 days.

A definite difference in the behavior of control eggs and centrifuged experimentals appears as early as the fourth week of diapause, and, as the age increases, more and more individuals leave the diapause state after being treated. By this procedure it is possible to estimate whether a given sample of eggs has been in diapause for a relatively long or short time. A more complete study of the influence of centrifuging on diapause is in preparation and will be published later. Table 1, however, is based on observations of over 7,000 eggs.

The centrifuging treatment was the only one used which had any consistent effect on the younger groups of eggs (Fig. 2); the following presentation will therefore concern itself only with the two older age groups shown.

Exposure to 0° C. for 6 days apparently had no effect on promoting development (Fig. 2). About the same number of individuals started postdiapause growth on removal as in the control lots. Ten degrees and 36° C. did tend to stimulate; about 60 per cent more eggs started to develop after these treatments than in the controls. Burdick (1937) states

TABLE 1*

DATA FROM CENTRIFUGING EXPERIMENTS ON SEVENTY-THREE DIFFERENT GROUPS OF EGGS

Age	Av. No. Devel.		Range	
	Contr.	Exper.	Contr.	Exper.
20–40	0	2	0	0–10
40–60	0	9	0	0–32
60–80	0	23	0	1–51
160–180	17	32	9–25	13–48
180–200	26	43	16–49	30–60

* For details see text.

that short exposures to 10° C. have a negligible effect in reducing hatching time when the eggs are replaced at 28° C. The samples he used, however, had been kept for only 21 days at 28° C. previous to the low-temperature treatment and are therefore comparable with lots 1 and 2 in Figure 2. Burkholder (1934) observed that breaking diapause by heat appeared to injure the eggs, but the writers found that the ones kept at 36° C. in the present series were apparently not harmfully affected and hatched normally.

The effect of storing the eggs in an atmosphere of oxygen for 6 days seems to be inhibitory. In the 158-day lot only one-third as many eggs began development within the 2-week period following the start of the exposure to the gas as in the control group. In the 153-day group the proportion was still less, approximately one-sixth. Figure 3, *A*, showing experiments on 11 lots of eggs of different ages, indicates the same result, that is, delay in the start of development after exposure to high oxygen tension.

Decreasing the metabolic rate through depriving the eggs of oxygen by keeping them in an atmosphere of nitrogen has the opposite effect. Thirty-six per cent more embryos started growth after this treatment than in the controls (Fig. 2, lot 4). Figure 3, *B*, shows a more complete age series of similar experiments with qualitatively the same result.

Lowering the oxygen tension by keeping the eggs covered with water seems to have no effect, at least during the 6-day interval tried (Fig. 2). The dissolved oxygen in the water may partially account for the difference between this lot and the ones kept in nitrogen.

It was thought that the breaking of diapause by low temperatures might be partly effected by the "piling-up" of carbon dioxide in the covered storage dishes. The action of a carbon dioxide atmosphere, however, seems to be retarding rather than stimulating (Fig. 2).

Disturbance of the water balance within the egg system has a pronounced effect on the initiation of development. Leaving the eggs in air on dry filter paper, although it had no influence on the two younger lots shown in Figure 2, changed markedly the number developing in the two older groups. One of these showed a 69 per cent increase over the controls, and the other 134 per cent. Although the eggs do not lose their shape in this mild dehydration, they take up water rapidly when the paper on which they rest is moistened, and it is perhaps this process that is significant. Schipper (1938) noted that in some cases "dehydration-rehydration may serve to shorten or eliminate the developmental block."

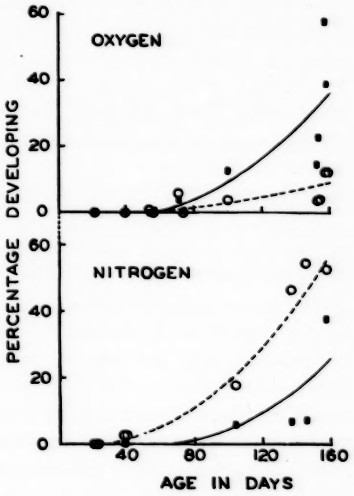

FIG. 3.—The action of atmospheres of oxygen and nitrogen on breaking diapause in grasshopper eggs (for details of exposures see text). Abscissae represent age of the eggs in days from laying; ordinates, percentage developing within a week after end of exposure; closed squares, controls; open circles, experimentals.

In the more vigorous dehydration obtained by placing eggs on filter paper wet with a ten-times isotonic sodium chloride solution enough water was removed to cause the eggs to lose considerable weight and some of them to appear shrunken. In this case some injury may have occurred, as all the eggs did not regain their original volume within a few days after being washed and replaced on filter paper moistened with distilled water. The treatment caused a definite decrease in the percentage of eggs which later developed, second only to that produced by the high oxygen tension and amounting to 51 per cent and 65 per cent of the controls in the two groups reported (Fig. 2).

From the results presented, it appears evident that the diapause condition decreases in stability as the period proceeds and that during its later stages abnormal treatment of various sorts may cause the eggs to resume active development. It is apparent also that during the earlier part of the period the egg is in a state quite resistant to any disturbing agent, and no method yet tried is even 25 per cent effective in breaking the block.

For purposes of speculation the five methods tried, which successfully break diapause in some of the older eggs, may be divided into two groups, the ones which may affect water balance and those that lower the metabolism. An increase in the water content of the egg seems to be associated with morphological development. It has been reported that the percentage of water in the whole egg becomes higher as prediapause and post-

diapause growth proceeds (Bodine, 1929; Slifer, 1938). Unreported observations of the writers also indicate that with increasing age in diapause and the accompanying decrease in stability, there is water uptake by the egg. It may be, then, that a change which brings about intake of water results in the breaking of diapause. Drying, centrifuging, and exposure to 36° C. may all lead to an absorption of water by the egg, but it is difficult to determine this accurately by weighing. In a group of eggs so treated some individuals will develop and, in doing so, take up water and increase in weight. Since it is impracticable to distinguish morphologically between these eggs and those still in diapause during the first 2 days of postdiapause development, they cannot be separated from the group, and their weight increase makes the gross weight of the group unrepresentative for the matter in question. Measurements of the weights of single eggs, whose development could then be followed, is also impracticable, because the degree of accuracy of the method overlaps the range of variation in the magnitudes being compared. Determination of the densities of individual eggs by a flotation method is being done at present and may give evidence bearing on this question.

Effects of a low level of metabolism are apparent in results of the experiments at a temperature of 10° C. and also in an atmosphere of nitrogen (Fig. 2). A question arises as to the lack of effect of 0° compared with 10° C. With an increased exposure at the former temperature, diapause is successfully broken; whether 10° C. is the more effective over a long period in destroying the block is yet undetermined.

Structure may possibly be another factor concerned in the potency of the block. Stuart (1935) reports that drying causes extensive disintegration of yolk cells within the egg. Centrifuging undoubtedly has a similar effect. If this action liberates from these cells a substance necessary for resumption of growth or destroys a factor that has been inhibiting development, the observed effects might occur. However, this conception is purely hypothetical.

More extensive investigations are under way at present in an attempt to understand more clearly the factors responsible for the maintenance and destruction of the diapause state.

SUMMARY

1. The stability of the diapause state in the egg of the grasshopper, *M. differentialis*, has been studied by subjecting eggs to various experimental conditions, including centrifugation, drying, temperatures of 10° and 36° C., and atmospheres of nitrogen, oxygen, and carbon dioxide. The number of individuals starting active development after the treatment was determined.

2. The stability of the diapause state varies with the age of the eggs, the younger ones being relatively resistant to all the treatments used, while the older ones are more easily induced to resume growth.

3. Treatments which were most effective in breaking diapause in old eggs are drying and wetting, centrifugation, exposure to nitrogen, and temperatures of 10° and 36° C.

4. Exposure to oxygen, carbon dioxide, and hypertonic sodium chloride solutions resulted in a smaller number of eggs breaking diapause than in the control groups.

5. A discussion is given of the possible correlation of the egg behavior with water balance, level of metabolism, or cellular structure.

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RESPIRATION DURING FLIGHT IN DROSOPHILA REPLETA WOLLASTON: THE OXYGEN CONSUMPTION CONSIDERED IN RELATION TO THE WING-RATE

(Three figures)

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Biological Laboratories, Harvard University

THE object of this study has been to obtain new information concerning the respiratory metabolism of an insect during flight. No previous paper known to the present writers treats quantitatively the respiration during flight of any dipterous insect, although Demoll (1927) inferred that the rate of respiration of mosquitoes (*Culex* sp.) must be increased in flight because they were then more rapidly

TABLE 1

DATA ON FLIGHT RESPIRATION RECORDED IN THE LITERATURE

Author	Species	Temperature (° C.)	O_2 Uptake at Rest (Cu. Mm. per Gram per Minute)	O_2 Uptake in Flight (Cu. Mm. per Gram per Minute)	O_2 Uptake in Flight / O_2 Uptake at Rest	Remarks
Tauchert	Apis mellifica	21	.....	1,217	.....	Running about very actively; sometimes flying
Kosmin, Alpatov, and Resnitschenko	A. mellifica	35	.....	4,600	.....	
		18	9	5,200	580	.....
		11	4	4,400	1,100	
Jongbloed and Wiersma	A. mellifica	21	32	1,679	52	Good flyers
		21	33	1,274	39	Poor flyers
Kalmus	Deilephila elpenor	20–22	2.8	104	37	.....
Raffy and Portier	Thais cassandra	20–25	6.7	3,605	540	Fluttering of wings induced by nicotine poisoning
	Sphinx ligustri	20–25	13.3	400	30	

poisoned by HCN than when at rest. Data on the respiration of flying bees (*Apis mellifica* L.) are given in the papers of Tauchert (1930), of Kosmin, Alpatov, and Resnitschenko (1932), and of Jongbloed and Wiersma (1934), while Kalmus (1929), and Raffy and Portier (1931) have studied the respiration of flying Lepidoptera. The literature has been reviewed in some detail by Jongbloed and Wiersma, so that it will be necessary to give here only a brief summary of the results obtained. This has been done in Table 1.

[1] James King of Irrawang Travelling Scholar of the University of Sydney.

In addition to these, mention should be made of the works of Parhon (1909) and Steidle-Zander (1921), who measured the oxygen uptake of swarms of bees, in which the insects, although probably not all flying, must have been in a state of considerable activity. Parhon obtained a value of 289 cu. mm. per gram per minute at 20° C., while Steidle-Zander reported at 20°–25° C. an oxygen uptake of only 34 cu. mm. per gram per minute.

Some of the evident disagreements in the literature are due to the wide range of variation which exists in the rate of respiration of different specimens, even of the same species; others are due to the different methods of measurement used by the various workers; and still others arise from the difficulty of estimating accurately the degree of activity of the specimens during the period of measurement. In the present study the rate of respiration has been recorded continuously, using a manometric method, while the rate of wing beat has been followed simultaneously with a stroboscope. It has been possible thus to make a direct comparison of the rate of respiration and the degree of activity and in this way to account for some of the variation in the rate of respiration of a single individual during flight.

The correlation of wing-rate and rate of oxygen consumption also allows a more accurate estimate of the energy requirements of the active flight muscles than could be made on the basis of past determinations. Such measurements are of particular interest, since the flight muscles of insects so greatly surpass most other contractile tissues in their capacity for repeated response at a rapid rate. In the species studied here the rate of wing beat is regularly of the order of 100–250 strokes per second, and fastened specimens have flown for periods of as much as 40 minutes, in the course of which the flight muscles will have performed several hundred thousand successive contractions.

METHODS

Specimens of *Drosophila repleta* Wollaston were selected at random from the same self-maintained culture that had been used in previous studies on wing-rate (Chadwick, 1939).

Oxygen uptake was measured in Warburg manometers having small cylindrical vessels of about 2.5 cu. cm. capacity. The insects were fastened in the center of the vessel and made to fly by the sudden removal from under the tarsi of a supporting platform. This was effected by means of a springboard arrangement, which was operated by an electromagnet situated outside the vessel.

The flies were mounted in the manner shown in Figure 1, being held by means of a small slip of paper attached with paraffin to the tip of the abdomen. The spring consisted of a small, flexible bristle (1), attached above to a small piece of glass (2), which was also provided with a projection (3) designed to carry the slip of paper supporting the insect. The platform (4) was of blackened paper, 6×4 mm., with a piece of iron wire fixed to the underside. The glass base (2) was fastened firmly by means of paraffin wax to the plug of the manometer at one side of the central bore. The variation in volume of the paper mounts for the insects and the amount of paraffin used in attaching them was small enough that it was not necessary to redetermine the volume of mount and spring for each specimen.

To insure rapid absorption of carbon dioxide, 0.06 cu. cm. of 10 per cent KOH was placed in the bottom of the vessel, as well as 0.04 cu. cm. in the side arm. All experiments were run in a water bath at a temperature of 25° C. The electric stroboscope was

placed directly above the manometer vessel. The insects were observed in silhouette through the bottom of the vessel, with the aid of a glass panel in the bath and a system of magnifying glasses and mirrors. The magnet was placed in a glass jar immersed in the

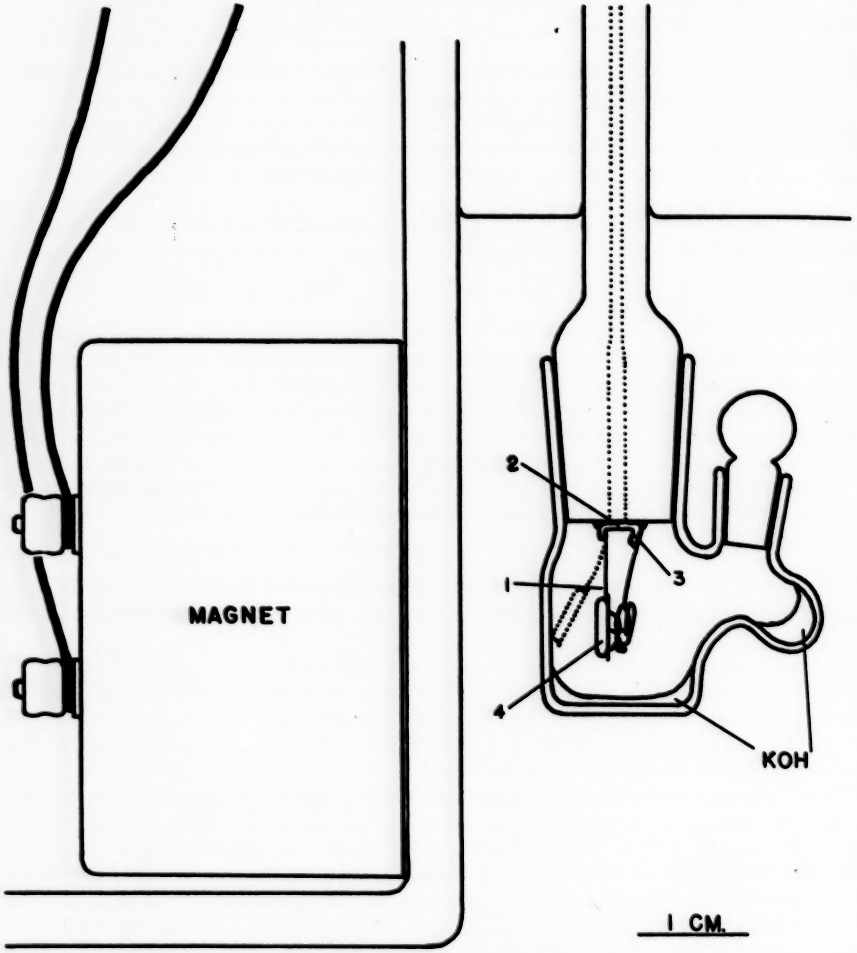

FIG. 1.—Apparatus used in studying the respiration during flight in *Drosophila*

water bath and arranged at a distance of not less than ½ cm. from the experimental chamber. An adequate circulation was maintained in the bath, so that there was a constant streaming of water between the two vessels. During flight, readings of wing-rate were made every 10 seconds, and manometer readings every 30 seconds. Under the

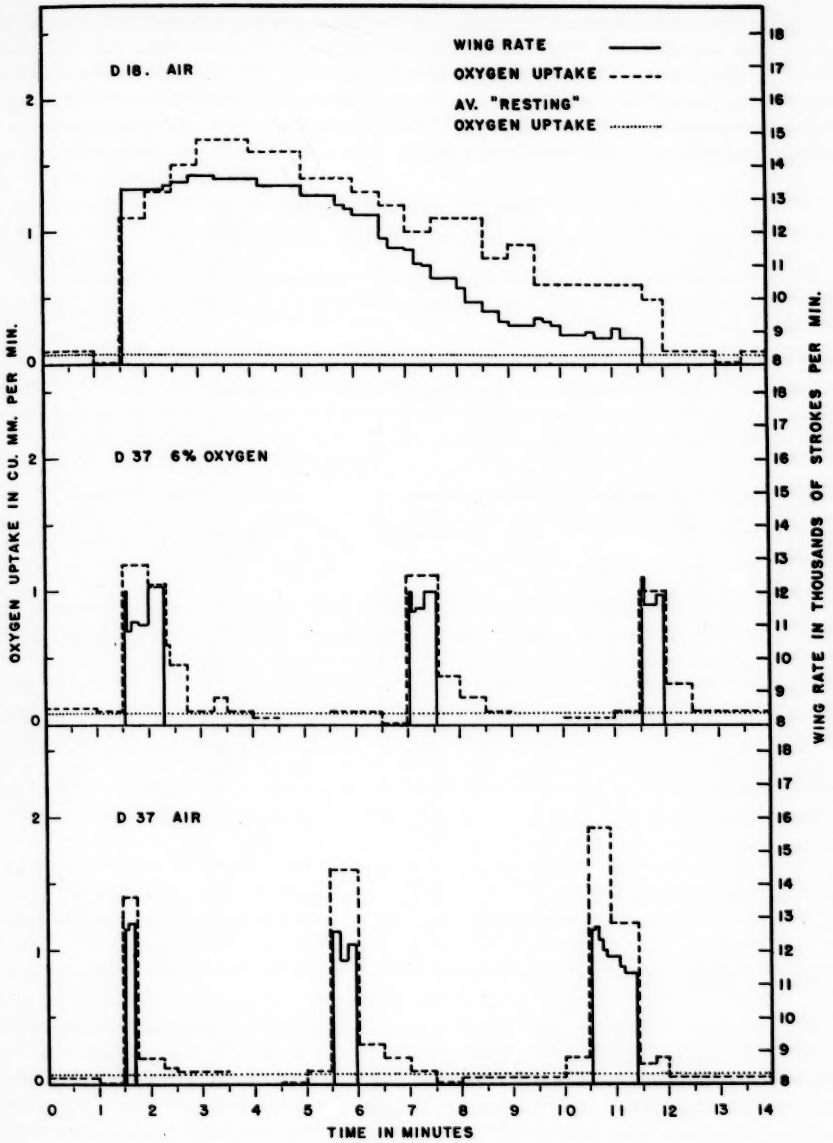
WING RATE
OXYGEN UPTAKE
AV. "RESTING" OXYGEN UPTAKE
D 18. AIR
D 37 6% OXYGEN
D 37 AIR
OXYGEN UPTAKE IN CU. MM. PER MIN.
WING RATE IN THOUSANDS OF STROKES PER MIN.
TIME IN MINUTES

FIG. 2.—Rates of oxygen consumption and wing movement during individual flights in *D. repleta* Wollaston. The resting level of oxygen consumption is an average of all the readings taken during resting periods on each insect.

conditions described, manometer excursions of from 1 to 5.5 mm. per half-minute, depending on the size and wing-rate of the animal, were obtained. The intensity of the "resting" metabolism was followed between flights and at the beginning and end of each experiment. Except in one instance, the insects were allowed to fly until they stopped of their own accord. In a series of blank controls it was determined that there was no change in pressure in the vessel as a result of running either the stroboscope or the magnet.

RESULTS AND DISCUSSIONS

MAGNITUDE OF THE GASEOUS EXCHANGES

Data were obtained on 16 insects which made a total of 76 flights ranging in duration from 15 seconds to 40 minutes 45 seconds. The results of several of these flights are presented graphically in Figure 2.

TABLE 2

FLIGHTS OF MORE THAN 1 MINUTE IN LENGTH*

Specimen	Weight (Mg.)	O_2 Uptake at Rest (Cu. Mm. per Gram per Minute)	Total Flying Time (Min.:Sec.)	Average Wing-Rate (Strokes per Minute)	O_2 Uptake during Flight		O_2 Uptake in Flight
					Cu. Mm. per Minute	Cu. Mm. per Gram per Minute	O_2 Uptake at rest
D3 ♀	2.4	29.5	3:50	10,310	0.77	337	11
D4 ♀	3.9	24.8	12:15	10,240	1.32	351	14
D9 ♀	5.2	25.5	12:10	12,770	1.75	349	14
D10 ♀	3.2	30.1	15:25	13,080	1.43	462	15
D13 ♀	4.3	29.0	7:20	12,270	1.66	419	14
D17 ♀	4.2	29.7	21:05	10,030	0.95	242	8
D18 ♀	2.9	23.6	11:30	11,930	1.12	399	17
D20 ♂	1.4	24.1	2:45	10,040	0.52	383	16
D21 ♀	2.1	23.0	32:20	9,540	0.78	382	17
D26♂	1.6	32.7	37:55	8,420	0.41	273	8
D31 ♀ (6 per cent O_2)	4.6	22.7	1:40	10,490	1.00	230	10
D31 ♀	4.6	22.7	55:35	10,020	1.11	256	11
D37 ♀ (6 per cent O_2)	4.1	20.6	3:20	11,820	1.20	302	15
D37 ♀	4.1	20.6	7:50	11,030	1.51	379	18
D38 ♀	2.8	45.7	9:55	11,880	1.33	499	11
Av.	.....	27.8	.....	10,920	1.12	351	13

* Insects flown in air unless otherwise stated.

The intensity of respiration at rest and during flight for the various animals is shown in Table 2. The average oxygen uptake is 28 cu. mm. per gram per minute during rest and 350 cu. mm. per gram per minute during flight, the increase being 13 times. During these flights the wing-rate averaged 10,920 strokes per minute. Both oxygen uptake and wing-rate may vary widely on either side of the average values. The greatest increase in oxygen uptake observed during flight was 26 times the resting value (D37, flying at a rate of 12,920 strokes per minute); the least, 5 times (D26, flying at a rate of 7,200 strokes per minute).

A number of flights were made in vessels containing only a small amount of water instead of KOH. In these experiments no significant change was noticed in the manometer reading, either during or after flight. The respiratory quotient thus appears to be 1, and carbon dioxide is given off by the insect as soon as oxygen uptake commences.

The average weight of the thoracic muscles is approximately 0.6 mgm., as determined by subtracting the dry weight of the thoracic skeleton after treatment with KOH from the weight of the freshly killed thorax.

From these data a number of calculations can be made, as follows: The average oxygen uptake per wing stroke, i.e., per single twitch (since each complete stroke represents a single contraction of each of two separate sets of muscles), is approximately 2×10^{-4} cu. cm. per gram of muscle. This represents the oxidation of 2.5×10^{-7} gm. glycogen and the liberation of 1×10^{-3} calorie of energy per gram of muscle for each contraction.

It is instructive to compare these figures with data obtained for frog muscle under somewhat similar conditions. Hill and Kupalov (1929) have obtained the following figures for an isolated muscle contracting isometrically in oxygenated Ringer's solution, the frequency of stimulation being such that there was no accumulation of lactic acid.

Average oxygen consumption per single twitch	8×10^{-4} cu. cm. per gram muscle
Glycogen oxidized per single twitch	11×10^{-7} gm. per gram muscle
Total heat production per single twitch	4×10^{-3} cal. per gram muscle

The average energy output per minute during flight in *D. repleta* is 11 cal. per gram muscle, at an average rate of contraction of 10,920 strokes per minute. In frog muscle contracting 23 times per minute the energy liberated each minute is 9×10^{-2} cal. per gram, and at the theoretically maximum rate of contraction of 37 times per minute in a "steady state" (Hill and Kupalov) it would be 15×10^{-2} cal. per gram.

A fly of average weight 3.5 mg. burning glycogen at the rate given above would use 0.1 mg. per hour of flight, i.e., 3 per cent of the body weight, or about 15 per cent of the weight of the wing muscles. Long flights must thus produce a considerable exhaustion of the total carbohydrate supplies of the body. It is possible that the amount of sugar in the blood is very high, as it is in the honeybee, in which Beutler (1936) found average blood sugar values of 2 per cent. According to Beutler, the total carbohydrate of bees of 100 mg. weight averaged 2.6 mg., and this supported 15 minutes of flight, during which the consumption of glucose was 8.5 mg. per hour. Jongbloed and Wiersma (1934) demonstrated a glucose consumption during flight of about 10 mg. per hour per bee. Given a total carbohydrate reserve in *Drosophila* similar to that in the bee, the total possible flying time should be rather less than 1 hour in a fly of 3.5 mg. weight. This agrees fairly well with the maximum flying times obtained in our experiments (Table 2: D21, D26, D31). The flights during which the respiratory quotient was followed were of relatively short duration (6 and 5 minutes), so we are unable to state whether or not substances other than carbohydrates act as sources of energy as the point of exhaustion is approached.

OXYGEN DEBT

It is to be noted that the oxygen uptake rises sharply at the beginning of flight and falls off rapidly immediately activity ceases (Fig. 2). There is a slight lag, however,

particularly noticeable at the end of flight, where the oxygen uptake remains considerably above the resting value for a period of usually 1–2 minutes after flight ceases. An explanation of this lag might be sought in any or all of the following factors: (1) lag in the apparatus, due to the time interval between production and absorption of carbon dioxide; (2) pressure changes in the vessel, due to heat production by the insect; (3) recovery processes in the muscles.

In order to test the first of these possibilities, a sleeve of Whatman filter paper, fitting around the inside of the vessel, was introduced in one series of flights. In spite of the great increase in absorbing surface of KOH provided in this way, no effect whatever on the magnitude or duration of the delayed oxygen uptake was observed. It seems, therefore, that whatever lag there is in the apparatus cannot be of much significance. This is not surprising, in view of the very efficient mixing of air accomplished by the wings of the flying insect.

That heat production by the insect is not responsible is shown by the fact that no change in the manometer reading was recorded in the experiments in which the KOH was replaced by water and the carbon dioxide not absorbed.

It is probable, therefore, that the continued high rate of oxygen uptake after flight ceases is due largely to the removal of lactic acid or some comparable substance accumulated during flight and, to a certain extent, to the restoration to the normal value of the oxygen tension of the tissues. During activity this must necessarily have fallen to near zero in order to maintain a maximum rate of entry of oxygen in a system which depends, at least in part, on gaseous diffusion. That we are concerned here with a true oxygen debt is further demonstrated by the fact that, in short flights of different durations, the ratio of the oxygen consumed in excess of resting requirements to the number of wing strokes is much more constant when the total excess of oxygen uptake (both during and after flight) is considered rather than the excess during only the actual flying time.

Table 3 shows the magnitude of the oxygen debt in a number of specimens. The average value from all flights is 0.18 cu. mm. The debt seems to be relatively constant for any one insect, regardless of the duration of flight, and is not obviously related to the body weight.

In experiments 31 and 37 the effect of a relative deficiency of oxygen supply on the course of oxygen uptake during flight was tested by flying the insects in a mixture of approximately 6 per cent oxygen in nitrogen. Difficulty was always encountered in inducing the insects to fly in this mixture, and the flights were invariably short (longest, 3 minutes, 20 seconds) and at a slower rate than was characteristic of the same animal in air. (It should be noted that this does not appear in Table 2, since the flights in air ranged over a wider series of rates and averaged lower than did the short flights in the 6 per cent mixture. The initial rates in air were always higher than those in 6 per cent oxygen.) The oxygen debt in these experiments did not differ markedly from that observed when the same individuals were flown in air.

The average value for the oxygen debt of 0.18 cu. mm. represents the amount of oxygen that would be used in making 1,000 contractions, corresponding to an accumulation of lactic acid in the muscles of about 0.2 per cent (assuming an oxidative quotient similar to that in frog muscle). In the experiments of Hill and Kupalov (*loc. cit.*) an isolated frog muscle was able to make, on the average, 700 contractions in nitrogen, accumulating lactic acid to a concentration of 0.3 per cent.

Whether or not the termination of flight is due to the fact that the concentration of lactic acid has reached a certain saturation value is not certain, although the relative constancy of the oxygen debt in different flights of a single individual points to this conclusion. But there certainly can be no constant rate of accumulation of lactic acid during flight in air, since the magnitude of the oxygen debt is independent of the length of

TABLE 3

MAGNITUDE OF THE OXYGEN DEBT*

Specimen	Length of Flight (Min.:Sec.)	Oxygen Debt (Cu. Mm.)
D9 ♀	9:40	0.23
	1:10	0.29
	1:20	0.17
	0:55	0.17
	0:50	0.17
	0:15	0.08
Av.		0.19
D13 ♀	1:35	0.54
	1:25	0.38
	2:00	0.69
	2:20	0.45
Av.		0.52
D17 ♀	9:45	0.26
	0:35	0.03
	5:10	0.15
	3:45	0.03
	2:25	0.04
	0:30	0.10
Av.		0.10
D31 ♀ (6 per cent O_2)	1:40	0.17
	0:35	0.29
	0:35	0.25
	0:30	0.15
	0:22	0.24
	0:28	0.29
	0:15	0.00
D31 ♀ (6 per cent O_2—*continued*)	0:15	0.17
	0:15	0.16
	0:15	0.21
	0:20	0.13
Av.		0.19
D31 ♀ (air)	40:45	0.23
	8:50	0.10
	6:00	0.11
Av.		0.15
D37 ♀ (6 per cent O_2)	0:50	0.17
	3:20	0.04
	0:35	0.18
	0:20	0.00
	0:57	0.06
	0:30	0.11
	0:15	0.04
	0:15	0.13
Av.		0.09
D37 ♀ (air)	6:15	0.00
	0:15	0.10
	0:25	0.11
	0:30	0.17
	0:55	0.05
	0:25	0.15
	1:35	0.08
Av.		0.09

* For weights see Table 2. Insects flown in air unless otherwise stated

flight. Limitation of the duration of flight in air may perhaps be explained more readily by the disappearance of some essential substance, such as carbohydrate. Certainly, the total possible flying time depends on the amount of reserve foodstuffs present, and it is possible that the termination of individual short flights is brought about by a temporary lack of substrate.

In the low-percentage oxygen mixture both length of flight and wing-rate are reduced, so that oxygen supply does seem to be a limiting factor. This is especially evident in the case of specimen D31, which, after 11 flights in 6 per cent oxygen, the longest of which

lasted only 1 minute 40 seconds, flew for successive periods of 40, 8, and 6 minutes in air. One would expect that for flights in 6 per cent oxygen the oxygen debt would be an expression of a maximal concentration of lactic acid in the muscles.

Failure to detect any difference in the magnitude of the oxygen debt between the flights in air and those in the 6 per cent oxygen mixture may be due to insufficient sensitivity of the apparatus, in view of the very small quantities involved, but it is also possible that the oxygen debt is an expression of a condition of exhaustion induced by two different means—in air by the irremediable disappearance of some substance connected with the oxidation process and in 6 per cent oxygen by the actual insufficiency of the oxygen supply.

OXYGEN UPTAKE IN RELATION TO WING-RATE

The wing movement may be considered theoretically to approximate a sine curve. Actually, up- and downstrokes are not quite symmetrical, and the retardation at the end of the stroke is rather greater than in a simple harmonic motion, but the experimental results may be expected to agree fairly well with a theoretical equation derived on this basis.

In a motion of this type the velocity at any point is equal to $v_0 \sin \omega t$, where v_0 is the maximum velocity at zero phase and ωt is the angle swept out in time t at an angular velocity ω.

Since the resistance to a body such as an insect's wing moving in air is proportional to the square of the velocity, over the range of velocities encountered, the work W done in any stroke should be proportional to the square of the average velocity of the wings (provided the angle of the wings in relation to their direction of movement does not vary from stroke to stroke), i.e.,

$$\begin{aligned} W &= Kv_0^2 \int_0^{2\pi} \sin^2 \omega t \cdot d\omega t , \\ &= Kv_0^2 \pi , \\ &= K' v_0^2 . \end{aligned}$$

But

$$v_0 = \omega \times \text{amplitude } (a) ,$$

and

$$\omega = 2\pi \times \text{frequency } (f) ;$$

therefore

$$v_0 = 2\pi a f ,$$

and

$$W = K'' a^2 f^2 ,$$

or for any given animal, since the amplitude is apparently constant,

$$W = K''' f^2 .$$

That is, the oxygen uptake per stroke should be proportional to the square of the wing-rate.

The experimental data are set out in Table 4, and, in Figure 3, f^2 has been plotted against the oxygen uptake per stroke for the three insects which flew the longest times. In the case of D26 and D31 the points lie along straight lines passing through the origin.

TABLE 4

OXYGEN UPTAKE PER STROKE IN RELATION TO WING-RATE*

Specimen	Number of Observations	Frequency (f) (Strokes per Min.)	$(f)^2 \times 10^{-6}$	Oxygen Uptake per Stroke (Cu. Mm. $\times 10^6$)	$\frac{(f)^2}{\text{Oxygen Uptake per Stroke}}$ ($\times 10^{-10}$)
D4	7	11,330	128	131	98
	10	10,400	108	130	83
	3	9,510	90	105	86
	4	8,680	76	104	73
D9	10	13,350	179	139	129
	14	12,530	157	127	124
	4	11,620	135	105	129
D17	4	12,270	151	113	134
	14	11,470	132	88	150
	9	10,680	113	78	145
	5	9,550	92	92	100
	2	8,430	73	89	82
	6	7,440	55	79	70
	2	6,550	42	99	42
D18	8	13,460	181	106	171
	3	12,690	161	90	179
	4	11,470	132	97	136
	2	10,310	108	101	107
	3	9,330	87	75	116
	4	8,880	79	60	132
D21	12	11,270	127	91	140
	13	10,270	105	82	128
	19	9,530	91	77	118
	9	8,510	72	68	106
	10	7,710	59	62	95
D26	22	9,380	88	56	152
	33	8,400	71	45	158
	23	7,590	58	35	166
D31	4	12,190	149	147	101
	14	11,440	131	125	105
	30	10,310	106	112	95
	48	9,540	93	97	96
	10	8,800	77	85	91
D37	6	12,640	160	151	106
	2	11,650	136	140	97
	2	10,550	112	135	83
	2	9,670	94	111	85
	5	8,640	75	92	82
D38	11	12,470	155	117	132
	7	11,510	133	102	130
	3	10,770	116	78	149

* Results averaged in intervals of 1,000 wing strokes per minute. Included are all insects which flew for more than 7 minutes, with the exception of D10 and D13, in which the range of wing-rates was very small. In the figures for oxygen uptake per stroke only the oxygen consumed in excess of the resting requirements is considered.

Number D21, however, does not agree with the theoretical equation. Of the insects listed in Table 4, D9, D26, D31, and D38 show a fairly close agreement with the expected relation; D37 agrees over part, at least, of its range of frequencies; while in D4 and D18 the scatter is rather too great to warrant a definite decision. Numbers D17 and D21 cannot be made to fit straight lines passing through the origin, and of these D17 shows so much scatter as to suggest that the points are supported by too few observations to be of much significance.

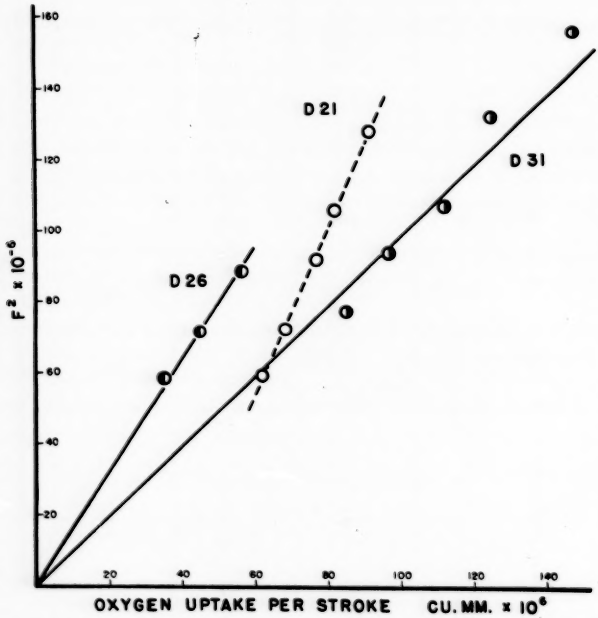

FIG. 3.—Relationship between the oxygen uptake per wing-stroke and the square of the wing-rate different specimens of *D. repleta* Wollaston.

We were not able to measure the amplitude during the course of flight, and, although there were no obvious changes, minor variation may have occurred in some cases. Since this factor enters the equation as the square, small variations would be magnified in their effect on the oxygen uptake per stroke. It is also possible that the pitch of the wings, and hence the mass of air moved per stroke, may be altered at some time during flight. Variations of this nature may have obscured the true relation between oxygen uptake per stroke and wing-rate in insects such as D21. The fact that the majority of the specimens, including those in which the figures are supported by the greatest number of observations, do agree with the theoretical relation suggests that the oxygen uptake per stroke is, in fact, proportional to the square of the wing-rate.

The constant K''', besides involving the amplitude of the stroke, must also include a number of factors depending on such quantities as body weight, area of wings, etc. It is

hardly to be expected that a constant relation between the one known factor among these—i.e., the body weight—and the oxygen uptake during flight could be demonstrated. And, in fact, when the body weight of the different individuals is plotted against average oxygen uptake per stroke *divided by* (average wing rate)^2, no relation does appear.

COMPARISON WITH PREVIOUS WORK

The percentage increase in respiration during flight that we have reported is considerably lower than the values previously obtained for flying insects. This may be due in part to the fact that the energy required for flight should be relatively less in a smaller insect, where the surface/mass ratio is larger. Although lower, our results are of the same order of magnitude as those of Jongbloed and Wiersma and those of Kalmus and the lower values reported by Raffy and Portier. It is to be noted that the increase of 540 times reported for one specimen by Raffy and Portier was obtained from an insect which "flew" for only 1 minute.

The average values which have been reported for respiration in bees, both at rest and in flight, are considerably higher than in other insects. The values obtained by Kalmus for *Deilephila* (Lepidoptera), on the other hand, are relatively very low. It is possible that in his experiments there was some loss of carbon dioxide in the paraffin oil over which the gases to be analyzed were collected.

The results of Kosmin *et al.* show much greater increases during flight than any others reported. These authors, however, seem to have used only one bee at each of the temperatures at which experiments were run, and the fact that, beyond stating that the insects were flown in Barcroft manometer vessels, they give no details of their methods makes the significance of their results difficult to assess. They state as a prerequisite for measuring the resting metabolism that "the animal must first be starved for 5–10 hours at 10°–11° C., until it is beginning to become stiff." It is not surprising that the resting values obtained under such conditions are very low.

Of course, no very rigid comparisons can be made when the figures under discussion depend on a purely arbitrary "resting respiration." In our experiments this value was measured with the animals held in a fixed position. Under these conditions they usually remained quiet, but there was a tendency to struggle at times. The results agree very well, however, with those of 2 experiments in which the flies were not fastened and the respiration was followed in darkness—conditions under which the insects remain without movement for long periods of time. The average of 17 experiments, ranging in duration from 20 to 200 minutes (i.e., excluding some of the values based on shorter experiments included in Table 1) was 24 cu. mm. per gram per minute, or 1,440 cu. mm. per gram per hour. Values obtained by Kucera (1934) for *D. melanogaster* Meigen at 26° C. were 1,660 cu. mm. per gram per hour for males and 2,380 cu. mm. per gram per hour for females.

SUMMARY

The average oxygen uptake found for *D. repleta* was 28 cu. mm. per gram per minute during rest and 350 cu. mm. per gram per minute during flight (average of flights of over 1 minute in duration). This represents an increase during flight of 13 times the resting value. The average wing-rate during the same flights was 10,920 strokes per minute.

The average oxygen consumption per wing stroke was approximately 2×10^{-4} cu. cm.

per gram of muscle, representing the oxidation of 2.5×10^{-7} gm. glycogen, the respiratory quotient being 1, and the liberation of 1×10^{-3} cal. of energy per gram of muscle.

Although there is some disagreement in the results, the data suggest that the relation between oxygen uptake per stroke and frequency of wing beat is such that the oxygen uptake per stroke is proportional to the square of the frequency.

The average oxygen debt, which was paid off during the first 2 minutes after the end of flight, was 0.18 cu. mm. This was not altered significantly when the same insects were flown in an atmosphere of 6 per cent oxygen in nitrogen, although both the duration of flight and the initial wing-rate were reduced in this mixture.

A comparison of our results with those reported previously indicates that the bulk of the evidence does not support the claim that extremely large increases in the rate of respiration occur during flight.

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THE RESPIRATORY METABOLISM OF THE FROG RETINA

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A REVIEW of the literature reveals a surprising lack of experimental work on the metabolism of the retina. For a number of reasons it would appear that the retina provides ideal material for studies on the metabolism of nervous tissue. In the first place, it consists primarily of nervous elements, originating embryologically from the brain; and, secondly, it can be readily removed from the eye with minimal injury to the cells and is thin enough to fall within the thickness generally required for respiratory studies, therefore obviating the necessity for slicing.

Among the earliest studies on the metabolism of the retina were those of Warburg and Nagelein (1929) and Nakashima (1929), who reported that light had no effect upon the rate of respiration in rats and fishes. Nakashima and Hayoski (1933) reported that the oxidation-reduction potential of suspensions or extracts of frog retina in Ringer's solution rises when the preparation is exposed to light and falls again when the light is removed.

Jongbloed and Noyens (1936) presented data in which they showed that the gaseous exchange in half-eyes of frogs was increased about 20 per cent in the dark. They attributed this increase in respiration to the resynthesis of visual purple. Chase and Smith (1939), however, reported that frog retina tested in light and dark showed no difference in the rate of oxygen consumption.

There seems to be no general agreement at the present time relative to the question of the effect of light on the gaseous exchange of the retina. Moreover, the work that has been done on the respiration of this tissue contributes relatively little to a general understanding of the complex chemical changes which undoubtedly take place under various conditions.

Considering the foregoing, it is obvious that the effects of dark and light should be further investigated before work can progress on the metabolism of the retina. Therefore, an attempt has been made, first, to study the rate of oxygen consumption in the frog's retina under controlled conditions of darkness and light and, secondly, to determine the amount of oxygen consumed per milligram dry weight of tissue under uniform illumination.

MATERIAL AND METHODS

The tissue used in this experiment was the retina of the frog, *Rana pipiens*. The frogs were obtained in groups of one hundred or more (approximately every three weeks) over a period of one year; and the data are presented with no regard to seasonal variation.

The retinas were prepared in the following manner: The frogs were decapitated, and their heads skinned; then the cornea and lens were removed. By means of curved forceps an approach was made into the eye between the choroid and the retina. A firm hold was obtained upon the optic nerve, and the retina was lifted from its position in the eye.

All experiments were performed with freshly prepared Ringer's solution brought to a

pH of 7.5 with Sorensen's phosphate buffer. The Ringer's solution was prepared each time from stock solutions of M/9 Baker analyzed grade of NaCl, KCl, and $CaCl_2$ in proportions of 100:1.5:1.

With the exception of a few experiments where a modified Fenn manometer was used, the data presented in this paper were obtained by the Warburg manometric method. The direct method described by Dixon for measuring oxygen consumption was used, and the manometers were filled with air. The rate of oxygen consumption in the series on the effect of light and darkness is expressed in millimeters change on the manometer index scale for comparable intervals of time, varying in different experiments from 5 to 15 minutes. In the second series the rate of oxygen consumption is expressed in cubic millimeters of oxygen consumed per milligram dry weight of tissue per hour. After each experiment the retinas were removed from the manometer flask and placed in weighing cells, dried for 10 hours at a temperature of 40° C., then weighed.

RESULTS AND DISCUSSION

Before pursuing the problem on the metabolism of the retina, it seemed desirable to establish whether the rate of oxygen consumption was different in darkness and in light. Data were obtained from dark-adapted retinas prepared in dim red light and from light-adapted eyes prepared in white light. The dark-adapted retina retained its deep pink

TABLE 1

THE EFFECT OF ALTERNATING LIGHT AND DARK ON DARK-ADAPTED RETINAS

Expt.	No. Retinas	Temp. (° C.)	Dark			Light*			Dark		Light*
1.....	5	25.3	6	8	8	7	7	7	6	7	6
2.....	5	25.3	6	6	6	6	6	5	6	5	6
3.....	5	25.3	5	6	7	6	5	6	6.5	6	4.5
4.....	5	25.3	6	6	7	6	6	6.5	6.8	5.2	6
5.....	5	25.3	5	4	5	5.5	4	4.5	6	4	4

* 2,500-meter candle; readings made at 15-minute intervals and given in terms of millimeters change on manometer index scale.

color when placed in the manometer flask, whereas those prepared in the light became completely bleached during the process of preparation. Manometers containing from 5 to 25 retinas were tested for various periods of time in alternating darkness and light. The light for illumination was produced by several 100–200-watt electric bulbs. The intensities used in the different experiments ranged from 750- to 2,500-meter candle-power at the level of the manometer flasks. In most experiments readings were made every 10 minutes, and the results were computed in relation to comparable time intervals. In all, 67 experiments were performed, with results which proved essentially the same for both dark-adapted and light-adapted retinas. No measurable differences in the rates of oxygen consumption were found at different intensities within this range.

The results from this series are given in Tables 1 and 2. The data presented in Table 1 are typical of all experiments on dark-adapted retinas; and Table 2 gives the results of a

similar series on light-adapted retinas. In no case could a consistent difference in oxygen consumption between dark and light be demonstrated.

Several experiments were modified so as to lengthen the total exposure to dark and light. This was done in the case of darkness to allow more time for the regeneration of the visual purple. There was no indication that this procedure altered the rate of gaseous exchange.

In some cases, where as many as 25 retinas were used in each manometer and readings were taken at 5-minute intervals, there was some indication of an increased oxygen consumption during the period immediately following exposure to the dark. However, these results were not obtained consistently with the Warburg manometer and appeared to warrant further investigation. To check this point a modification of the Fenn manometer, having a scale reading of 0.001755 cc. per centimeter on the index capillary, was employed. A light intensity of 2,500-meter candle-power at the level of the flask was used. This intensity of illumination was sufficient to bleach the retina completely within

TABLE 2

THE EFFECT OF ALTERNATING LIGHT AND DARK ON LIGHT-ADAPTED RETINAS

Expt.	No. Retinas	Temp. (° C.)	Light*			Dark				Light*			Dark
1	7	25.3	5.5	5.8	4.7	6.3	4	5	4.8	5.3	4.9	5.5	4.5
2	7	25.3	4	5	4.4	5.6	3.5	4.5	5.1	3.5	5	4	5
3	7	25.3	4	4	5.4	5.6	3.5	4.6	4.6	5.1	4	4.3	4.1
4	7	25.3	4.6	4.7	4.5	4.8	4.5	4.6	5	5.2	4.2	4.9	5
5	7	25.3	5.5	5.3	5.8	6.0	5.5	5.2	5.9	5	5.7	5.5	5.4

* 2,500-meter candle; readings made at 10-minute intervals and given in terms of millimeters change on manometer index scale.

10 minutes. From 1 to 4 retinas were used in each manometer, and readings were made every 5 minutes in alternating 20-minute periods of darkness and light. A very slight increase in the rate of oxygen consumption was usually obtained, lasting for about 10 minutes after the first exposure to darkness; however, subsequent changes from light to dark rarely gave any deviations from the value obtained for light.

With the foregoing data indicating that variations of light intensities, ranging from complete darkness to approximately 2,500-meter candle-power, exert no marked effect on the rate of respiration of the frog retina, a second series of experiments was designed to determine the amount of oxygen consumed by the retina. The oxygen consumption of from 2 to 10 retinas was determined during periods ranging from 2 to 4 hours at a light intensity of 750-meter candle-power. The solution in which the retinas were tested was the same as in the previous set of experiments. Approximately 400 retinas were used in 93 experiments. Table 3 presents the results of three groups of representative experiments taken at different times during the course of the investigation.

The data presented in the first two groups of experiments were obtained from paired retinas, whereas in the third group the retinas were removed and placed together before transferring them to the manometers. When retinas from different frogs are tested together, the rather large variation in metabolism so often found between different frogs is

not so apparent. The mean oxygen consumption for approximately 400 retinas tested in this series was found to be 3.22 ± 0.634 (S.E.) cu. mm. per milligram dry weight of tissue per hour. This compares quite favorably with the metabolism of brain tissue and ranks the retina as one of the most actively respiring tissues in the body.

TABLE 3

OXYGEN CONSUMPTION OF FROG RETINA AT 25°.3 C.

Expt.	No. Retinas	Dry Wt. (Mg.)	Oxygen Consumed*	Expt.	No. Retinas	Dry Wt. (Mg.)	Oxygen Consumed*	Expt.	No. Retinas	Dry Wt. (Mg.)	Oxygen Consumed*
1	2	3.5	2.95	1	2	4.1	3.04	1	5	11.1	3.18
2	2	4	2.18	2	2	4.1	2.91	2	5	11.3	3.21
3	2	4	2.44	3	2	4.0	4.12	3	5	10.2	2.89
4	2	3	3.69	4	2	3.5	2.96	4	5	12.1	3.08
5	2	3.2	3.48	5	2	3.5	3.91	5	5	12.3	3.50
Total	10			Total	10			Total	25		
Av.			2.95	Av.			3.39	Av.			3.16

* Oxygen consumed in cubic millimeters per milligram dry weight of tissue per hour.

SUMMARY AND CONCLUSIONS

1. The metabolism of the frog retina was studied with the Warburg method and a modified Fenn manometer.

2. The rate of oxygen consumption of the retina was determined for alternating periods in the dark and in the light.

3. The Warburg method reveals no consistent difference in the rate of oxygen consumption in the dark as compared with the light.

4. The data obtained with a Fenn manometer indicate a very slight increase in oxygen consumption for approximately 10 minutes after a light-adapted retina is exposed to darkness, followed by return to a value which remains constant during exposure to light.

5. The retina of the frog consumes an average of $3.22 \pm$ cu. mm. of oxygen per milligram dry weight of tissue per hour.

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THE EFFECT OF THYROID FEEDING ON OXYGEN CONSUMPTION OF THE GOLDFISH

(Two figures)

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THE influence of thyroid medication in raising the basal metabolic rate in mammals is well understood. The increase in metabolism is, indeed, so regular in some mammals—as, for example, man—that quantitative relationships could be standardized. Boothby and Sandiford (1928) state that each milligram of thyroxin injected increases metabolism by 2.8 per cent in myxedematous patients. From mammalian work the concept has arisen that a function of the thyroid hormone is to regulate the basic rate at which the processes of metabolism proceed. It is natural and easy for such a generalization to be extended from mammals to lower vertebrates, for structurally and chemically the thyroid gland appears to be much the same in all vertebrates. Yet, when this generalization is examined critically, it is seen to be based on little concrete evidence. The primary basis should rest on evidence that thyroid medication does increase the metabolic rate of the resting organism or conversely that thyroid removal lowers it. For the warm-blooded vertebrates this may be accepted as proved. The literature makes but scanty reference to any of the cold-blooded forms other than the amphibia. The reason for the extensive work in amphibia lies in the relation of this problem to the much-studied problem of the thyroid stimulation or metamorphosis. The usual interpretation (at least among American and English workers) of the work on the effect of thyroid on the metabolism of the metamorphosing amphibian has been that the metabolism is greatly accelerated but that this acceleration is not primary to the metamorphic activity (Helff, 1926). In 1934 one of us (Etkin) studied the oxygen consumption of tadpoles in normal metamorphosis. The results showed that during the growth phases of early metamorphosis the metabolism of the individual increased only in porportion to its growth. During the first days of the climax of metamorphosis, when the tadpole lost as much as 30 per cent of its former weight, the oxygen consumption of the animal likewise dropped and approximately to the same extent. Thus, per unit gross weight the metabolic activity seemed to remain constant. But it was further pointed out that the loss of weight in the animals in the climax of metamorphosis was almost entirely to be ascribed to loss of water from the tissues and to the loss of intestinal contents, the dry weight of the tadpole remaining about constant. When the entire desiccation curve was considered, it was evident that per unit dry weight the rate of metabolism of the tissues fell during all periods of metamorphosis and most rapidly during the metamorphic climax. Since the thyroid was believed to be active during metamorphosis and especially during climax (for general

[1] The authors are indebted to Dr. G. K. Noble, director of the Laboratory of Experimental Biology at the American Museum of Natural History for the use of the facilities of the laboratory during the progress of this investigation. The investigation has also been aided by a grant from the Josiah Macy Fund. Assistance in the preparation of these materials was furnished by the personnel of Work Projects Administration Official Project No. 65-1-97-23 W.P. 10.

discussion see Etkin, 1936), it is evident that this conclusion is not consistent with the concept of metabolism control through thyroid activity. A critical re-examination of previously published evidence on thyroid and metabolism was then made, and it was found that the interpretation of other authors was based upon the fact that per unit gross weight the metabolism of animals artificially metamorphosed by thyroid show a distinct increase. However, the metabolism per individual dropped as in Etkin's data. The increase per unit weight reported by the previous workers was at least largely to be ascribed to the loss of water and intestinal contents rather than to actual increase in metabolism per unit dry weight. According to some data of Romeis (1920), there is a greater or speedier loss in water and intestinal contents in artificial than in normal metamorphosis. Whatever discrepancies appeared between Etkin's data on normal metamorphosis and the results reported by various experimenters on artificial metamorphosis seem to originate from this source. It seemed clear, therefore, that an interpretation of a thyroid effect on the metabolism of such animals was uncalled for and certainly equivocal.

Since 1934 there appears to be only one new study bearing directly on this problem, that of Wills (1936). In his study of oxygen consumption in various amphibia during development he reported increases in rate during metamorphosis. The chief concern of Wills's paper was with a study of metabolism in relation to sex differentiation. Though introducing several important advances in technique, particularly the use of protein nitrogen as basis for calculating the rate of metabolism, his treatment of the metamorphic problem suffers so much from a lack of appreciation of the literature and details of this process itself that it is difficult to evaluate his evidence. For example, though describing his techniques in considerable detail, he made no mention of the treatment of intestinal contents. Etkin previously pointed out the importance of this, at least for the frog, since at the beginning of the metamorphic climax in the tadpole the intestinal contents are evacuated. Thus an error which, in weight and presumably in nitrogen, may amount to more than 20 per cent is introduced, by not taking this loss of inert material into consideration. Even aside from the error of intestinal contents, his data on *Ambystoma* and *Rana pipiens* are too scanty to allow any clear inference or statistical evaluation. Indeed, the trend lines drawn through the points in his Figures 2 and 3 seem hardly consistent with the points themselves. Wills's few results on metamorphosis as given in Table 5 of his paper seem to us to be entirely consistent with the data and interpretation Etkin presented and indicate no fundamental increase in metabolism in the tadpole during metamorphosis.

It is evident that the problem in the metamorphosing amphibian must always be complicated by the profound morphological changes that take place under thyroid influence. The question of metabolism in cold-blooded animals would best be examined, therefore, where separated from metamorphosis. Henschel and Steuber (1931) have made an extensive study of thyroid injection and feeding in normal and in thyroidectomized frogs. Their findings were entirely negative; no effects of the experimental procedures on metabolic rate were found. This was apparently a thorough study, and its conclusions may be accepted as definitive for the adult frog. Drexler and Issekutz (1934) concluded that in a fish, a reptile, and two amphibia thyroid treatment does not raise the metabolic rate. Unfortunately, with regard to any particular form the evidence given is entirely too variable and scanty to justify much confidence in the conclusion. Perhaps, taken as a whole, the work justifies greater credence, but here again we are faced with personal opinion rather than with objective evidence.

The only other work (through 1939) known to the authors on the question of thyroid and metabolism of cold-blooded adult animals is found in the papers of Taylor (1937, 1939) on the salamander, *Triturus torosus*. He there developed the idea that the metabolism effect of thyroidectomy is at least partly secondary to the disorganization of the animal's nutrition. His data on oxygen consumption in thyroidectomized animals, calculated per gram total weight, show an initial slight increase for the first 16 days, followed by a more marked decrease 21–32 days after operation. It is not entirely clear whether, in view of the edema shown by the thyroidectomized animals, the oxygen consumption per animal or per unit dry weight would show any corresponding decrease or increase at any time. In a personal communication Taylor has suggested that there might not be any appreciable difference calculated per unit dry weight following thyroidectomy. Taylor thought to produce the hyperthyroid condition by transplantation of two pairs of thyroids. The results recorded indicate a profound drop in metabolism from the eighth to the twenty-fourth days and a great increase in the forty-sixth to the fifty-third days. The justification for considering that mere implanting of glands will induce hyperthyroidism may be questioned, especially since instead of the expected increase in molting these animals showed a decided decrease. In any case the irregularity shown by the results seems to the present authors to make it very difficult to give a physiological interpretation. This situation in the literature indicates the desirability of a new study of the relation of the thyroid to the metabolism of cold-blooded vertebrates. Such a study should (1) deal with an animal in which possible thyroid effects on metabolism are not accompanied by special morphological effects and (2) obtain data by a method permitting the establishment of standard conditions and make the data extensive enough to permit definitive statistical treatment.

Accordingly, we have set about to make such a study and have investigated the effects of thyroid on the metabolism of fishes. A preliminary report of our results with thyroxin administration in a single species of marine fish was reported earlier (Root and Etkin, 1937). It is our purpose here to report the results of a more extensive study on the common goldfish.

MATERIAL AND METHODS

A stock of about 150 goldfish was purchased from a commercial dealer. They were kept in large aquariums at 23°–25° C. for 3–4 weeks before experimental use. During this period they received the regular control food, which consisted of a mixture of dried shrimp, liver, lettuce, and oatmeal in about equal parts by weight.

The apparatus used was a continuous-flow type modified after Keys (1930*a*). A number of the modifications were copied from the apparatus used by Dr. Frank Schuett at the University of Chicago. In this apparatus a continuous flow of water passes through the animal container. Oxygen content of water samples was determined by the micro-Winkler method. From the difference between the oxygen content in the outflow from the animal chambers and that from a blank tube and the rate of flow, the oxygen consumption of the fish may be determined.

The structural details of our apparatus may be briefly summarized as follows: Cold New York City tap water was run through block-tin coils surrounded by running hot water. This served to warm the tap water, which then passed over a baffle system by which the oxygen content was brought to saturation at room temperature. The water was then led through a cotton filter to an overflow reservoir, which served to maintain a

constant head of water as a source of supply to the animal chambers, since it was kept overflowing at all times. From the reservoir the water was led through block-tin coils in the water bath to bring its temperature exactly to that of the bath. A distributor pipe then sent the water to each of 11 glass chambers. The animal chambers consisted of 8-inch lengths of 1½-inch glass tubing. The inlet was through the rubber stopper at one end, and the outlet in the corresponding place at the other end. The outlet was guarded by a fine-mesh wire filter. The outlets led through sections of capillary glass tubing to the bottom of the collecting bottles. The collecting bottles had a capacity of 35–45 cc. and a narrow neck only slightly larger in diameter than the capillary tubing. Such an arrangement meant that the overflow water from the collecting bottles must pass upward through a narrow space between the tubing and the neck of the bottle and would thus offset the possibility of any contamination of the sample with room air. We have checked this very simple collecting system with more elaborate ones and found it to be satisfactory. The collecting bottles were all arranged on a sloping shelf, and each sat in a metal tray that served to collect the overflow and concentrate it at one point, where it could be passed into a measuring flask for rate-of-flow determination. The rate of flow through the chambers could be regulated for the entire setup by a screw clamp at the outlet of the overflow reservoir or for individual chambers by varying the length of the capillary tubing. The water bath was provided with a cover that excluded almost all light from the animal chambers. Since in respiration measurements an experimental and a control group were always run simultaneously for comparison with each other, exact control of temperature was not deemed necessary. Temperature control was effected through the laboratory water-mixing valves. Only rarely did the temperature vary as much as ½° C. during the course of a 3-day respiration period. Through the course of the work the temperature varied from 24° to 27° C., but in the great majority of the readings (except low-temperature ones) it was about 26° C.

What must be emphasized here are the advantageous characteristics of this type of apparatus for the problem at hand. Perhaps most important of these is the possibility presented of allowing the animals to remain completely undisturbed in the chamber for days, while a series of readings is taken of their oxygen consumption. As will be seen later, this is a most important condition to be established if any standard metabolism measurements are to be attempted. Continuous-flow methods further avoid the difficulties and uncertainties inherent in the closed-system type because of the accumulation of the products of metabolism and the changing oxygen and carbon dioxide tensions. From the results of such studies as that on experimental confinement described later, it seems apparent to the authors that manometric methods involving shaking of the animal are out of the question because of the disturbance to the animals. In the present study the rate of flow for each fish was so adjusted as to result in the extraction by the fish of about half the oxygen of the water. This permitted the greatest accuracy, since the differential between animal and blank water was large, without, however, allowing the oxygen tension to drop to the point where it affects appreciably the rate of oxygen consumption (Keys, 1930*b;* Wells, 1935).

A point deserving of note is that the size of our collecting bottles, animal chambers, and the method of determining the rate of flow differ from those used by Keys and by Wells, both as regards the simplicity and ease of manipulation and as regards the kind of data yielded. From a calculation of the volumes of water involved it can be seen that our method gives a measure of the rate of oxygen consumption during the last few min-

utes before the sample was removed, whereas in Keys's method an average result over a period of several hours is given. The relative desirability of the two methods would of course depend upon the problem at hand.

The recent careful investigations of the Winkler method of determining dissolved oxygen (Allee and Oesting, 1934; Wilder, 1937) have shown the reliability of the method in dealing with uncontaminated waters. The suitability of the present water for this method has been checked by paired comparisons with the permanganate modification of the Winkler method, which is designed to remove interfering substances from the water. The results obtained by both methods were in good agreement.

The present report will discuss the results of five different types of treatment. These are (1) a preliminary determination of the effects of confinement, starvation, and external disturbances upon the oxygen consumption of the fish, (2) a paired comparison of the oxygen consumption of thyroid and control-fed fish, (3) a group comparison of such, (4) a comparison of the effect of a drop of temperature on the oxygen consumption of normal and thyroid-fed animals, and (5) effect of thyroid feeding on weight. The details of treatment in each series might better be discussed in connection with the results. In general, however, the following methods were pursued with regard to feeding and the taking of respiration readings. The control animals were fed two or three granules of fish food daily. The experimentals were fed on alternate days with fish food and with a single desiccated thyroid tablet (Burroughs Wellcomb & Co.)—each tablet representing 1 gm. fresh gland substance or 2/5 gr. U.S.P. powder. Each tablet was about equal in volume to the ration of fish food, so that the experimental animals received about half of their food supply as desiccated thyroid tablet.

Before being put into the respirometers, the animals were starved for 24 hours. Respiration readings were begun 18–24 hours after introduction. Two to 4 readings per fish were then taken daily for 2–4 days. The animals were left undisturbed and not fed during this period. After each such period of treatment the animals were returned to their aquariums for feeding periods of a week or more.

EXPERIMENTAL ANALYSIS

I. EFFECT OF EXPERIMENTAL CONFINEMENT AND LIGHT STIMULUS ON OXYGEN CONSUMPTION

A group of 20 animals was divided equally between two aquariums and fed control food for 1 week. Immediately after the last feeding the first 10 animals were introduced into the respiratory chambers, and O_2-consumption measurements immediately begun. Eleven readings in all were taken over a period of 54 consecutive hours, 6 the first day, 2 the second day, and 3 the third day. On the morning of the third day, after the first respiration test, the black screen covering the apparatus was removed, and the animals exposed for half an hour to the light of a 100-watt bulb held just over the tank. Immediately after turning on the light, a reading was begun (i.e., the rate of flow determined), the sample bottles being removed between 15 and 20 minutes after the light was turned on. The cover was then replaced, and another reading taken 3 hours later.

In Figure 1 the results are given. It can be seen that from the first to the second hour the oxygen consumption increased slightly on the average but thereafter fell steadily during the rest of the first day (to 6 hours after introduction). At the twenty-fourth, twenty-eighth, and forty-eighth hours the rate was distinctly lower than on the first day

but seemed to have reached a fairly steady state about 35 per cent lower than the initial rate. This drop is clearly a real drop not to be ascribed to chance variation of the figures. The effect of the light stimulus was a 10 per cent increase in oxygen consumption followed by a decrease of about 20 per cent. The variability of the material is such that these smaller differences are not clearly significant. It can be seen that there is a tendency for the variability to be somewhat less on the second and third day than on the first.

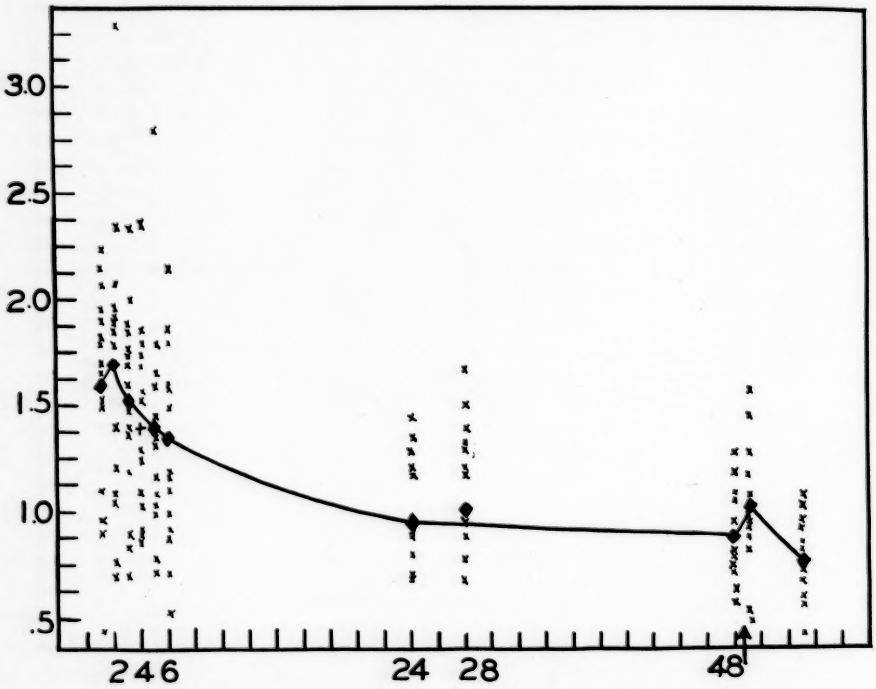

FIG. 1.—Rate of oxygen consumption in relation to time after introduction into respirometer; effect of light. Abscissae, hours after introduction; ordinates, cubic centimeters O_2 consumed per fish per hour. Arrow indicates time at which light was turned on.

The most noteworthy points brought out in this experiment are (1) the drop in metabolism in the first day and from the first to the second day, (2) the relatively steady metabolism during the second and third day, and (3) the small effect of the light as a disturbance. These results are very close to those of Wells (1932) in regard to the time factor.

It is, therefore, evident that for the standardization of metabolism reading it is very desirable that readings be taken only after the animals have been many hours in the apparatus. We have followed the practice of allowing 18–24 hours for the attainment of the steady state.

II. PAIRED COMPARISONS OF THYROID- AND CONTROL-FED FISH

After the preliminary weeks of adjustment to laboratory and food conditions the animals were selected in pairs, the members of each pair being as much alike as possible. The animals were placed individually in evaporating dishes, fed control food for a week, and then subjected to a preliminary period of oxygen-consumption measurements. In some cases two or three such periods were established before the experimental feeding was introduced. One member of each pair then was fed desiccated thyroid on alternate days, and the other continued on control food. Respiration tests were made as often as possible after the beginning of thyroid feeding, and the experiment continued as long as the animals seemed in good condition.

TABLE 1

RATIO OF OXYGEN CONSUMPTION IN PAIRED EXPERIMENTAL AND CONTROL FISH

No.	Ratio Exp./Cont. Prelim.	Ratio in Experimental Periods				
		3–10 Days	11–17 Days	18–24 Days	25–31 Days	32–38 Days
1	0.98±0.03	.....	.....	.....	.....	0.85±0.02
2	1.52±0.09	.....	.....	.....	.....	1.12±0.14
3	1.32±0.05	1.90±0.13	1.18±0.06	.....	.....	.....
4	0.93±0.06	1.11±0.09	.....	.....	.....	.....
5	1.77±0.09	.....	.....	1.82±0.07	1.85±0.11	.....
6	0.78±0.05	.....	1.05±0.07	.....	.....	.....
7	0.99±0.03	1.26±0.07	.....	1.14±0.05	.....	.....
8	0.65±0.03	0.68±0.03	0.74±0.07	.....	.....	.....
9	0.91±0.08	0.99±0.03	0.93±0.04	1.44±0.13	1.11±0.11	1.04±0.06
10	0.88±0.03	1.23±0.06	.....	.....	.....	.....
11	1.25±0.10	1.16±0.08	1.89±0.21	.....	.....	.....
12	1.64±0.14	0.95±0.06	0.88±0.06	0.98±0.08	0.90±0.06	0.83±0.07
13	0.90±0.05	1.07±0.07	.....	.....	.....	.....

The results of the paired comparison experiments are summarized in Table 1. It can be seen that 13 cases are available for analysis, covering the period from 3 to 38 days after the beginning of experimental feeding, as well as the preliminary periods. Since the animals had been matched as to size, etc., the ratio of oxygen consumption in experimental and control animals was usually near 1. The figure given in the table is the average ratio for a series of 6–21 separate determinations made during the period in question. With each average is given its standard error. The experimental periods are classified into 5 groups according to the number of days after the beginning of experimental feeding that the readings were taken.

Even a cursory examination of the table shows that there was no constant alteration of metabolism associated with thyroid treatment. In some cases the ratio of the experimental to control decreased, in others it increased; but in the majority it showed no significant alteration. A statistical evaluation reveals that 10 of the 26 experimental periods show a significant deviation, 4 in a negative, and 6 in a positive, direction. (A probability of 0.01 was taken as the lower limit of significance.) Further evidence of the failure of thyroid materially to affect the metabolism of our fish is to be seen in the fact that where more than one experimental reading is available on one pair of fish there is no correlation

between the effect in one period and that in another. Thus the third pair shows a significant rise in the first week, followed by a fall in the second, which, however, is not statisti-

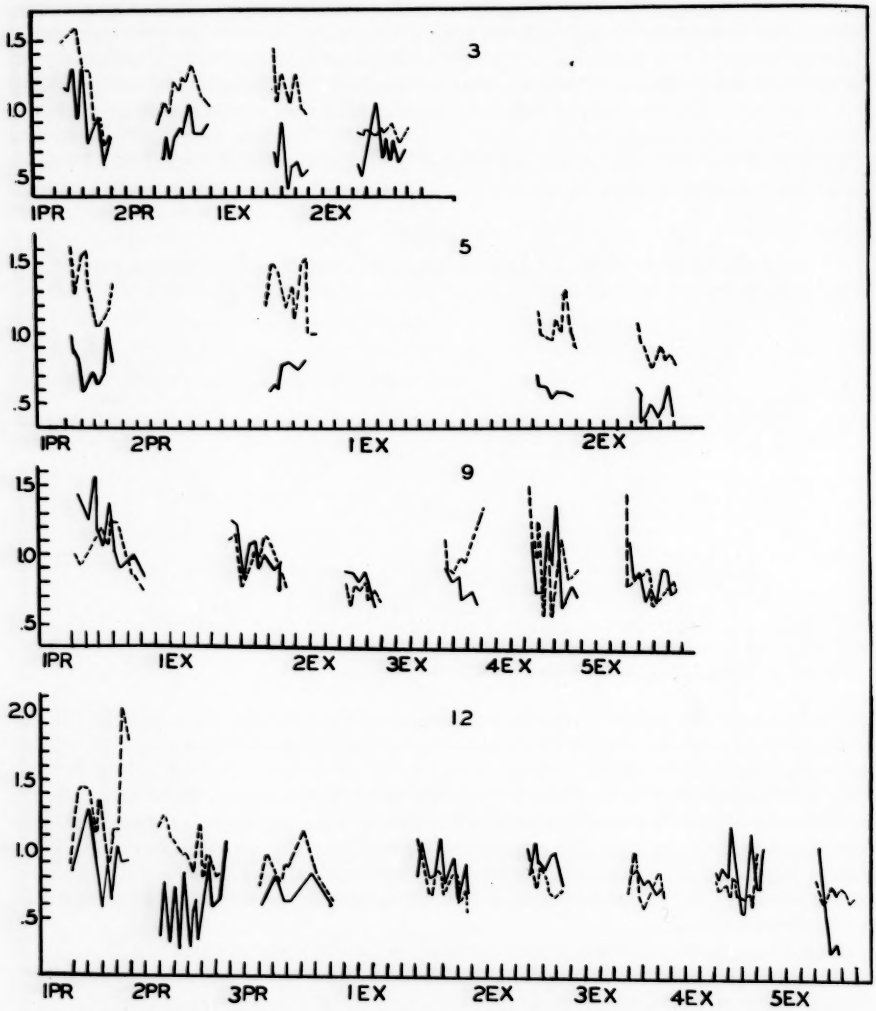

FIG. 2.—Rate of oxygen consumption in relation to thyroid feeding in four sets of paired comparisons. Ordinates, cubic centimeters O_2 per fish per hour; abscissae marked off in days. *PR*, preliminary period; *EX*, experimental period.

cally significant (see also Fig. 2). Only the seventh pair showing an increase and the twelfth showing a decrease exhibit any constancy of effect, and, of course, they do not

agree in direction. Finally, it should be noted that the evidence does not indicate any trend in the data with time, the periods showing significant variations being about evenly dispersed. Even after 5 weeks of thyroid feeding no specific effect appeared. Thus, the evidence indicates that the thyroid feeding had no demonstrable effect on the metabolism of the fish. In the seventh and twelfth cases, mentioned above as showing constancy in effect, the effect did not increase with time.

One point in regard to statistics is deserving of some comment. It might be objected that 10 deviations out of 26 tries is rather high on the calculated basis that each had a probability of 0.01. But it must be recalled that the variance upon which the calculation of probability is based is taken from the data within each experimental period. The variability from one period to the next is not taken into account. The large number of significant deviations may be taken to indicate that a real change often takes place in the relative rate of metabolism of the experimental and control animals, our previous remarks being to the effect that this real change is not ascribable to the influence of thyroid.

TABLE 2

OXYGEN CONSUMPTION IN RELATION TO TIME AFTER BEGINNING OF THYROID FEEDING*

Time	A		Ratio	B		Ratio
	Control	Exper.	Exper./Control	Control	Exper.	Exper./Control
Preliminary...	0.99±0.05	1.07±0.06	1.08	1.15±0.08	1.13±0.07	0.98
4 weeks.....	0.80±0.04	0.84±0.05	1.05	1.11±0.06	1.15±0.07	1.04
6 weeks.....	.....	.....	.....	0.91±0.08	0.93±0.05	1.02
7 weeks.....	0.66±0.04	0.73±0.05	1.10	.....	.....	.....
9 weeks.....	0.71±0.04	0.70±0.05	0.99	.....	.....	.....

* Oxygen consumption given as cubic centimeters O_2 per fish per hour.

This is, perhaps, best appreciated in an examination of a graphic representation of the data for some of the pairs of animals as in Figure 2. In the first group (pair No. 3) the solid line representing the control animal showed a distinct drop after the first experimental period but came up again after the second period of experimental feeding. The experimental, on the other hand, remained about the same or rose slightly after the first period and fell at the second. It is thus apparent that in this particular case the significant change seen in Table 1 after the first thyroid feeding is to be ascribed more to the fall in the control rather than to a direct rise in the thyroid-fed animal. The results plotted in the other cases reveal again cases where a relative change occurred in the metabolism of experimental and control which, though clearly real enough, cannot be ascribed to any metabolism effect of thyroid. In the second pair plotted (pair No. 5) it is apparent that both animals clearly tended to fall off in metabolic rate with the passage of time, in this case at much the same rate. A general tendency in this regard was observed in all our animals, as, for example, in the graphs here given and in the data of the group experiments in Table 2.

Thus far our interpretation of results leads us to the conclusion that by the technique applied in this study no effect of thyroid feeding on oxygen consumption could be demonstrated. Such a conclusion is, of course, all that could properly be expected in the interpretation of negative evidence. Yet it is interesting to ask whether we have any internal

evidence as to how accurate our study is, how large a deviation it would detect, or, stated conversely, how large a difference which, if present, would have been clearly revealed by our methods. It is possible to make a calculation of the amount of deviation which, with data of the variability of our material, would give a significant difference from the mean of the preliminary period. Such a difference may be expressed as percentage of the mean.[2]

The average figure found for this percentage was 34 per cent. In 18 of the 26 cases it did not exceed the average, being as low as 12 per cent in 1 case. It is thus apparent that under the conditions of these experiments any factor which raised or lowered the metabolism of a particular fish by as much as 34 per cent would be definitely detectable in a single period of average variability. A trend in a group of cases such as here presented would, of course, be apparent even if the influence of the factor responsible were considerably less than 34 per cent.

As we have seen above, no influence or even definite trend was found in the thyroid-fed animals. We may conclude, therefore, not only that our results showed no influence of thyroid feeding on metabolism, but, more, they showed that any influence that may have escaped detection in the present experiments could have been of only minor significance with an effect of considerably less than 34 per cent.

III. GROUP COMPARISON OF THYROID- AND CONTROL-FED FISH

It was felt that the paired comparison experiments described above suffered most from the fact that the animals did not usually survive well for more than a few weeks. The frequent confinements in the respirometers under starvation conditions were judged the chief cause of this, though the animals also did not seem to remain completely healthy, merely as a result of the necessary isolation in evaporating dishes. A group experiment was therefore designed to overcome these deficiencies.

In this experiment 40 animals from the stock aquariums were selected and grouped into 4 groups of 10 animals each. The selection was made so that 2 sets of experimental and control groups could be made, the members of each set being of about the same size. All animals were fed control food for 2 days and then placed in the respirometers for a set of preliminary readings. They were then returned to their aquariums, and the differential feeding of control and experiment groups was begun. The experiment was continued for 7 or 9 weeks, with oxygen-consumption readings being taken at the fourth, seventh, and ninth week. The results are given in Table 2.

It should be remarked that the average given for each period was found by using as individual values the mean of the 5 or 6 determinations for each individual fish. These figures were then averaged, and the standard error calculated as recorded in Table 2.

[2] The formula used in calculating the statistical significance of the difference of the means of the preliminary and experimental periods is taken from Fisher (1925). It is

$$t = \frac{(x_1 - x_2)\sqrt{N_1 + N_2 - 2}}{\sqrt{S_1 + S_2}} \sqrt{\frac{N_1 N_2}{N_1 + N_2}}.$$

The probability P was derived from "Students" distribution for the calculated t and the appropriate degrees of freedom. For the calculation of the size of deviation that our methods would reveal, the t corresponding to the assumption of a probability of 0.01 was obtained from the table, and the equation solved for the value of $(x_1 - x_2)$. This value was then expressed as percentage of x_1. The data on variability used in this calculation was that supplied by the readings for the preliminary period and for the experimental period in question.

The significance of this method is that the measure of variability given represents the variability between fish and is not mixed with measures of the variability of successive readings of the same fish. It can be seen that in each series there is a tendency for the O_2

TABLE 3

THREE EXPERIMENTS ON THE EFFECT OF A DROP IN TEMPERATURE IN THYROID-FED AND CONTROL GOLDFISH*

Day	Temperature (° C.)	Thyroid-Fed	Ratio High/Low	Control-Fed	Ratio High/Low
		Experiment A			
1	25	0.69±0.13	.....	0.65±0.11	.....
1	25	0.64±0.09	.....	0.62±0.11	.....
1	14.5	0.21±0.05	0.32	0.19±0.05	0.30
2	14.2	0.25±0.03	0.38	0.21±0.03	0.34
2	15.5	0.23±0.02	0.35	0.21±0.03	0.34
3	15	0.26±0.04	0.39	0.25±0.04	0.38
3	15.2	0.26±0.04	0.39	0.26±0.04	0.41
		Experiment B			
1	25	1.12±0.10	.....	0.76±0.05	.....
1	25	1.21±0.09	.....	0.84±0.16	.....
2	24	1.06±0.09	.....	0.74±0.18	.....
2	13.5	0.41±0.06	0.36	0.27±0.05	0.35
2	13	0.24±0.04	0.21	0.26±0.03	0.33
3	14.5	0.38±0.02	0.37	0.28±0.05	0.36
3	15	0.28±0.07	0.25	0.27±0.03	0.35
4	14.5	0.34±0.03	0.30	0.28±0.02	0.36
		Experiment C			
1	24	0.73±0.07	.....	0.67±0.03	.....
1	26.9	1.03±0.06	.....	0.82±0.06	.....
2	25	0.87±0.07	.....	0.83±0.07	.....
2	13	0.29±0.02	0.33	0.26±0.03	0.34
2	15	0.18±0.06	0.21	0.22±0.06	0.29
3	15	0.41±0.09	0.47	0.27±0.03	0.35
3	15	0.34±0.03	0.39	0.30±0.04	0.39
4	15	0.33±0.03	0.38	0.27±0.03	0.35

* Oxygen consumption given as cubic centimeters O_2 per fish per hour.

consumption to drop off considerably with the passage of time. This in spite of the fact that the animals remained very active and healthy in appearance throughout. It will be noticed, however, that no significant difference in the amount of drop appeared as between the thyroid- and nonthyroid-fed animals. The ratio between these dropped some-

what in the first series, at least at the ninth week, and rose slightly in the second series. Both changes, however, are clearly within the limits of error of the experiment. It is, therefore, apparent that in these experiments running for as long as 7–9 weeks of thyroid feeding no significant effect of the experimental feeding could be detected.

IV. TEMPERATURE EFFECT

Huxley (1929) has speculated on the possibility of the thyroid's acting as a temperature buffer to reduce the accelerating action of heat on the rate of metabolism of cold-blooded animals. According to this notion, the activity of the gland (or perhaps the reactivity of the tissues to the hormone) decreases as the temperature increases; thus the loss of thyroid stimulation when the temperature is raised decreases the total effect of the temperature increase. The possibility becomes apparent that the failure we experienced in detecting any effect of thyroid on O_2 consumption could possibly be ascribed to the rather high temperature (26° C.) at which the experiments were run. We, therefore, determined to study the effect of a drop of temperature on the O_2 consumption of control- and thyroid-fed animals.

A group of experimental animals were paired with controls and fed according to the experiments previously described. In the first experiment (Table 3, *A*) the animals surviving from the group experiment were used. In these the experimentals had been fed thyroid for over 7 weeks; in the other experiment the animals were differentially fed for 3 and 2 weeks, respectively. The animals were introduced into the respirometers in the afternoon, allowed to rest undisturbed over night, and the next day two readings of O_2 consumption were taken. In the first experiment the temperature was quickly lowered in the entire apparatus, and another reading was taken between 2 and 3 hours thereafter. In the later experiments the drop in temperature was delayed another day, giving time for an early morning determination at high temperature and two determinations at lower temperature. Readings were continued to the third day after dropping the temperature. An examination of Table 3 shows that the drop of approximately 10° C. in temperature resulted in a drop to approximately $\frac{1}{2}$ in O_2 consumption. This drop was pretty well maintained for the 3 days of the experimental period, though in the first experiment a slight tendency of the values to rise might be inferred. It is, however, abundantly clear that the thyroid-fed animals behaved in all respects very much like their controls, no constant difference appearing at any time. In short, the temperature coefficient of O_2 consumption in these animals did not seem to be affected by the period of thyroid feeding that had gone before.

V. EFFECT OF THYROID FEEDING ON WEIGHT

It would, of course, have been desirable to follow the weight changes on the same animals as were used in the respiration studies. This, however, proved impracticable, since it was found difficult to keep the fish in good condition and the added handling involved in weighing clearly was detrimental to them. Weighing the fish used in respiration studies was therefore soon abandoned, and recourse was had to a separate experiment on this point. An added point to be established by a study of this kind was the question of the nutritive adequacy of the control and thyroid feeding. Ten groups of 5 fish each were set into aquariums. These groups were made up in pairs, each member of the pair having about the same average and range of weight. The animals were fed for 1 week on control food and then weighed. One group of each pair was then subjected to the thyroid-feed-

ing schedule, and the other group served as control. Weighings were again made at the fourth, ninth, and sixteenth weeks after the preliminary period. A standardized handling was developed to cut down as much as possible on the variability. This consisted of shaking the excess water off the fish by three swings of the arm and then weighing in water in a tarred cardboard container.

The results of this experiment are shown in Table 4. Inspection of the figures shows that, in general, the control fish maintained their weight pretty well but did not gain or lose much. The experimentals showed much the same behavior. Occasionally as in group 10 there was some loss, or as in group 4 consistent gain, but, on the whole, the picture closely duplicates that of the controls. It is thus clear that a diet consisting of approxi-

TABLE 4

EFFECT OF THYROID FEEDING ON WEIGHT IN GOLDFISH*

Duration of Thyroid Treatment	Set 1		Set 2		Set 3		Set 4		Set 5	
	Control	Exper.	Control	Exper.	Control	Exper.	Control	Exper.	Control	Exper.
Prelim.	12.61	12.56	15.03	14.85	11.15	11.14	11.15	11.21	14.33	14.20
4 weeks	12.55	12.34	14.85	16.03	11.69	11.75	11.30	11.30	13.80	14.34
9 weeks	12.49	12.29	14.06	15.51	11.77	11.64	11.22	11.19	13.51	12.85
16 weeks	12.48	12.48	13.65	15.22	11.82	11.52	11.28	11.16	13.84	14.38

* Figures give average weights in grams of the 5 animals in each group.

mately one-half of a dried thyroid tablet does not lead to any marked alteration in growth or weight maintenance as compared with our control food. It is clear that no marked difference in the nutritional value of the two types of diet appeared. No adjustment of the respiratory trends for weight changes or for other nutritional factors is called for.

DISCUSSION

The negative results of thyroid feeding upon the oxygen consumption and the weight of goldfish here reported are not consistent with the commonly held opinion that thyroid substance is a general stimulant to cell metabolism. They are, however, in agreement with the results of the two European investigators mentioned in the introduction and are further consistent with the interpretation one of us had previously made of the more extensive literature on amphibian metamorphosis in relation to thyroid and metabolic rate. We feel justified, therefore, in concluding that the effect of thyroid on general metabolism of mammals and birds may not properly be extended to any of the cold-blooded animals that have been worked on, including fish and adult amphibia.

It may always be said that such negative results as are reported in this paper may be due to a technical failure of one kind or another. We believe that we have disposed of the objection that our technique was not capable of detecting a small effect by showing in a number of ways that the apparatus was capable of yielding clear-cut results on effects amounting to from 10 to 30 per cent. If a diet containing as much thyroid as our experimental feeding does not produce a detectable effect under these conditions, it seems hardly possible to think of the thyroid as of any considerable physiological importance in the regulation of the rate of oxygen consumption in these animals. Mere illumination of the

animals without further disturbance produces a detectable physiological effect which thyroid feeding does not do.

Perhaps a more significant objection to an interpretation that denies a role in metabolism regulation to the thyroid in cold-blooded animals lies in the fact that we (and all other workers) fed mammalian thyroid to nonmammalian forms. It would indeed be very interesting to compare nonmammalian forms in their response to their own and to mammalian thyroid. Until this can be done, however, we must be content with pointing out that Henschel and Steuber (1931) found no metabolic effect of thyroidectomy in frog; that mammalian thyroid is effective in other regards on cold-blooded animals, i.e., amphibian metamorphosis, amphibian and reptilian skin shedding; and that Artundo and Solari (1933) found that anterior pituitary extracts effective on the dog were ineffective in raising the basal metabolic rate of toads (despite the fact that it induced thyroid stimulation in the toad). What evidence exists negates the idea that the thyroid plays any considerable role in regulating the rate of metabolism in cold-blooded animals.

The role of the thyroid in homoiothermal regulation has long been the subject of considerable investigation. The absence of a metabolic effect of the thyroid in cold-blooded animals might be taken as indicating the special significance of the thyroid for temperature regulation. A general discussion of this problem seems out of the province of the present paper, but the somewhat analogous notion, ingeniously developed by Huxley (1929), that the thyroid serves as a physiological buffering device against changes in temperature in cold-blooded vertebrates deserves further consideration. It was felt that, if this were indeed the case, a difference in the ability of the fish to adjust itself to a sudden lowering of the temperature might be expected as between control and thyroid-fed animals. As noted above, we were unable to detect any difference between the control and experimental fish in this regard. We do not regard this test as a sufficient exploration of the possibilities of the idea. Yet the results do not encourage any confidence in its further development.

In view of the failure of the evidence from many sources and of many varieties to show any metabolic rate regulating effect of the thyroid on cold-blooded animals we are inclined to the view that such a relationship is peculiar to the physiology of the homoiotherms. The physiological importance of the thyroid in the economy of the cold-blooded vertebrates lies perhaps in the specific tissue responses as metamorphosis, skin-shedding, etc., which are known in some animals.

SUMMARY

A study of the effect of thyroid feeding on the oxygen consumption of goldfish was made. Desiccated mammalian thyroid was fed to the extent of about half the food supply of the experimental animals. The methods of metabolism determination permitted the attainment of a high degree of standardization and of adequate statistical evaluation. The following results were found.

1. During the first 6 hours after introduction into respirometers the oxygen consumption of normal undisturbed goldfish fell steadily but during the second and third days maintained a steady state about 60 per cent of the initial value. Exposure to light seemed to raise the metabolism about 10 per cent.

2. Animals fed thyroid tablets to the extent of half their food supply did not reveal any increased metabolism even after 9 weeks. A statistical evaluation of the results indicates that an effect of considerably less than 30 per cent would have been clearly revealed.

3. The effect of a drop of temperature on oxygen consumption was the same in control- and in thyroid-fed animals.

4. Thyroid feeding had no effect on the weight of fish even when continued for 16 weeks.

The results are interpreted as supporting the notion that the metabolism-accelerating effect of thyroid substances as found in mammals is absent in goldfish.

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NUTRITIONAL STUDIES OF PARAMECIUM MULTIMICRONUCLEATA
II. BACTERIAL FOODS

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PURELY experimental studies on the growth and reproduction of *Paramecium* have been limited to the last fifty years. Observations of isolated cultures of these organisms, however, were made as early as 1830 by Ehrenberg, who followed the multiplication of 2 single individuals in clear water through a 10-day period. During the first 6 days "these polygastric Infusoria existed without their reproductive power being developed," but by the end of the tenth day these animals had multiplied to such an extent that it was impossible to count them. In 1860 Balbiani estimated that a single *Paramecium* would produce 1,384,416 offspring in the course of 42 days. He obtained this figure by multiplying together the progeny of several successive subcultures which had been isolated in hay infusions.

In 1888 Maupas criticized the observations of these early investigators in that the importance of the food factor in their studies on growth and reproduction was overlooked. Discussing the nutrition of protozoa, he said: "Perhaps by employing the methods of Pasteur for the pure culture of Schizomycetes, one would succeed in finding one still more appropriate to the needs of the Ciliates." However, he did not make any attempt to find such a food organism, nor have many others in their experimental studies of protozoa.

Hargitt and Fray (1917) were the first to use bacteriological methods in making a detailed study of the food requirements of *Paramecium*. The bacteria chosen as food organisms were those which appeared to be dominant at different periods of an aging hay infusion. Of the 30 bacteria isolated in pure culture, 11 were partially identified. From their findings Hargitt and Fray concluded that mixed cultures of bacteria are, as a rule, far superior to a single strain as a diet for *Paramecium*. The conclusions of Phillips (1922) on feeding *Paramecium* known bacteria substantiated those of Hargitt and Fray.

Cleveland (1928*a*) criticized the work of Hargitt and Fray and that of Phillips by stating:

> They have attempted to grow the paramecia in too few pure cultures of bacteria for the investigation to have much value. Had they used more pure cultures of bacteria, it is very probable that quite different results would have been obtained. Instead of finding, as they did, that *Paramecium* grew better in mixed cultures of certain bacteria than in pure cultures, it is most likely that they would have found it grew better in pure cultures of certain bacteria—perhaps far more abundantly than in mixed cultures.

He also pointed out that their work was limited by the lack of most of the usual bacteriological facilities.

Owing either to the suggestion of Cleveland or to the realization that bacterial feeders can thrive on a single strain of bacteria, recent investigators studying paramecia experimentally have usually employed only pure cultures of bacteria as food for this ciliate. However, a survey of the literature to determine a satisfactory food for paramecia

TABLE 1

SUITABILITY OF BACTERIA AS FOOD FOR SPECIES OF *Paramecium* AS REPORTED BY VARIOUS INVESTIGATORS

Investigator	Paramecium	Culture Medium	Food Organism	Suitability as Food
Hargitt and Fray, 1917....	caudatum	Organic	Micrococcus flavus	−
	aurelia	Organic	Bacterium plicatum	−
			Bacillus Raveneli	−
			B. dendriticus	−
			B. guttatus	−
			B. fluorescens	−
			B. flavescens	−
			B. subtilis	±
Phillips, 1922.....	aurelia	Organic	B. coli	−
			B. cereus	−
			B. proteus	−
Philpott, 1928.....	aurelia	Organic	B. pyocyaneus	+
	calkinsi		B. enteritidis	−
	caudatum			
Pringsheim, 1928.....	caudatum	Organic	B. proteus	+
			Azotobacter	−
Chejfec, 1929.....	caudatum	.....	B. coli	+
Raffel, 1930.....	aurelia	Organic*	B. candicans	+
Damerow, 1931.....	caudatum	Organic	Tubercle bacilli	−
Losina-Losinsky, 1929 and 1931.....	caudatum	Organic	B. fluorescens liquefaciens	−
			B. coli communae	−
			B. subtilis	+
Hetherington, 1934.....	caudatum	.....	Achromobacter pinnatum	+
Phelps, 1934.....	aurelia	Organic*	Erythrobacillus prodigiosus	+
Giese and Taylor, 1935....	multimicronucleata	Organic*	Pseudomonas ovalis	+
Johnson, 1936.....	caudatum	Inorganic	Escherichia coli	−
			Aerobacter aerogenes	−
			B. subtilis	+
			Ps. fluorescens	−
			Serratia marcescens	−
			Staphylococcus aureus	−
Giese, 1938.....	multimicronucleata	Organic*	Ps. fluorescens	±
			B. subtilis	±
Johnson and Hardin, 1938..	multimicronucleata	Inorganic	Ps. fluorescens	+
De Lameter, 1939.....	aurelia	Organic*	Flavobacterium brunneum	+
			B. niger	+
			B. cereus	+
			B. prodigiosus	−
			B. coli	−

* Considered organic when either lettuce or algae is used in connection with the bacteria.

shows that there may be but little agreement as to the suitability of a given food organism. Table 1 summarizes the findings of investigators as to the food suitabilities of various bacteria introduced as pure cultures into an organic or inorganic culture medium. From this table it is evident that different investigators do not always report similar findings as to the food qualities of a specific bacterium. Such a lack of understanding of the basic problem of protozoan nutrition in a form so much investigated as *Paramecium* is unfortunate.

Studies of the foods of such bacterial feeders as *Tritrichomonas fecalis* and *Glaucoma ficaria* have been made by Cleveland (1928*b*) and Johnson (1936), respectively, and have aided materially in our understanding of the nutritional requirements of these protozoa, as a large number of bacteria were tested as food organisms in each case. Luck, Sheets, and Thomas (1931) have reported nutritional studies on *Euplotes taylori*, feeding on a very limited number of isolated pure strains of bacteria, as has Hetherington (1933) for *Colpidium campylum* and *C. colpoda*. The importance of the food factor was clearly stressed by Jahn (1934), when he pointed out the numerous factors affecting the growth of protozoa. And Hammond (1938), who has made a review of the literature dealing with the role of foods in relation to allelocatelytic phenomena, indicates the significance of understanding the food factor.

Recently it has been demonstrated that *Pseudomonas fluorescens*, a bacterial food of *Paramecium multimicronucleata*, can be qualitatively and quantitatively standardized, facilitating reproducible results in experimental studies on this ciliate (Leslie, 1940). Using these same techniques, it was decided to try to determine the relative suitability of some 30 species of bacteria as food organisms for *P. multimicronucleata*.

MATERIALS AND METHODS

The standardization of experimental cultures of *Paramecium* involves the use of (1) Osterhout's non-nutrient culture medium, (2) a pedigreed strain of food bacteria qualitatively controlled as to age and quantitatively measured by the wire-loop method, and (3) controlled optima for all physical factors normally considered.

The food organisms selected for the present investigation were pedigreed strains obtained from the bacteriology department of Stanford University. All these foods were qualitatively standardized by cultivating the bacteria for 24 hours at optimum temperatures on slants of the commercial Difco Bacto nutrient agar except for the species of Mycobacteriaceae, which were cultured for 48 hours because of their slower growth. The reason for cultivating the bacteria either 24 or 48 hours before use was to insure a large number of living bacteria in each food suspension. The necessity of daily subculture of the bacteria required that the purity of all the cultures be regularly checked throughout the course of the experiment; none of these became contaminated. At the time of feeding the paramecia, two level loopfuls of the qualitatively standardized (24- or 48-hour-old) bacteria were removed from the slant with a 1-mm. platinum loop and suspended in 5 cc. of the sterile salt solution serving as the culture medium.

The ratio of the volume of the culture medium to the number of paramecia at the time of subculture was constant at 1 cc. to 20 animals. Sterile volumetric pipettes were used to carry 1 cc. of the bacterized culture medium into clean ground Columbia culture dishes of 1-cc. capacity, in which the ciliates were grown as isolation cultures.

The paramecia were subcultured daily by isolating 20 with a sterile-mouth pipette from a similar culture started 24 hours earlier and grown in a glass moist chamber main-

tained at 26°–27° C. Daily counts of the animals in each 1-day-old subculture were made under a dissecting microscope, with counting facilitated by the use of a mechanical hand counter. From the total number of progeny produced over a 4-week period the average daily fission-rate of the ciliates was determined in each of the foods tested. These rates served as an index of the suitability of the various food organisms.

No dust-protecting hood was employed during the daily subculture of the ciliates into the bacterized inorganic culture medium, as is usually the case when an organic culture medium is used. As will be pointed out later, no bacterial contamination resulted from this procedure. This was to be expected, as W. H. Johnson (1933) previously reported that this medium will not support the growth of the bacterial inoculum.

The control food for these experiments was *Ps. fluorescens*, which was cultivated for 4 days on the nutrient agar before being suspended in the salt medium. Unfed paramecia in a 1-cc. volume of sterile salt solution were also subcultured daily, and these permitted a rough control in comparing the suitability of the various food organisms. The animals used in this study were taken from a mass culture of *P. multimicronucleata* which Professor W. H. Johnson has had growing in his laboratory at Stanford University for many months at 25° C. in Osterhout's inorganic culture medium on *Ps. fluorescens*.

EXPERIMENTAL DATA

The species of bacteria chosen as possible food organisms for *P. multimicronucleata* are nonpathogenic representatives of several different families. Although the number of selected bacteria was as large as possible to work with satisfactorily, there were still too few species tested to make comparisons between the families. The present investigation was designed merely to determine the various bacteria as food organisms under standardized experimental conditions.

In order to determine the suitability of these bacteria the degree of acclimation of the ciliate to them was used as a criterion, the degree of acclimation being the average daily fission-rate which the paramecia maintained in the standardized bacterial suspensions over a 4-week period. If any of the subcultures failed to become acclimated to a particular bacterium and consequently died out during the course of the experiment, such cultures were replenished by washed animals from the mass culture. Thus all the parental cultures were assumed to be comparable.

Owing to the number of parallel cultures maintained, it was possible to carry only a single-animal culture in each of the different food suspensions. Hence the suitability of the various bacteria as food organisms has been estimated according to the average daily fission-rate of the paramecia in single-line cultures as follows:

1. Division-rate 0.0–0.9 ..... food organism POOR
2. Division-rate 1.0–2.0 ..... food organism GOOD

The division-rate was calculated from the progeny of the 20 paramecia subcultured daily. If after 24 hours of cultivation the progeny numbered 40, the division-rate of the animals during that period would be 1.0, since each of the organisms had apparently divided once. Had the number of offspring been 60 or 80 during the same period, the division-rate would have been 1.5 or 2.0, respectively. From the average daily number of individuals present in each subculture the average daily fission-rates were calculated and the suitability of the food organisms estimated. Table 2 summarizes these findings according to (*a*) classification number of the bacteria, which is that of the bacteriology de-

partment of Stanford University, (*b*) scientific name of the bacterium, (*c*) total number of progeny, (*d*) duration of the experiment in days, (*e*) average number of offspring produced per day from each subculture, (*f*) average daily fission-rate of the paramecia, and (*g*) rating of the suitability of the bacteria as food organisms.

TABLE 2

SUITABILITY OF BACTERIA AS FOOD FOR *Paramecium multimicronucleata* CULTURED IN AN INORGANIC MEDIUM

Cat. No.	Bacterium	Total Progeny	Days	Av. No.	Division Rate	Suitability
D-2	Bacillus mycoides	832	27	30	0.5	Poor
D-10	B. cereus	1,303	29	44	1.1	Good
D-20	B. megatherium	1,967	29	67	1.6	Good
D-21	B. mesentericus	894	27	33	0.6	Poor
D-28	B. niger	1,068	29	36	0.8	Poor
D-41	B. terminalis	942	27	34	0.7	Poor
D-39	B. subtilis	655	15	43	1.0	Good
D-40	B. subtilis	322	14	23	0.1	Poor
K-11	Escherichia coli	1,034	29	35	0.7	Poor
K-12	E. coli	774	30	25	0.2	Poor
A-5	Aerobacter cloacae	1,711	29	59	1.4	Good
A-6	A. aerogenes	1,372	29	47	1.1	Good
Al-2	Erwinia carotovora	1,508	29	51	1.2	Good
Al-1	E. atroseptica	789	30	26	0.3	Poor
C-1	Alcaligenes faecalis	1,670	29	57	1.4	Good
T-6	Proteus vulgaris	1,224	27	45	1.1	Good
Z-1	Serratia marcescens	1,260	27	46	1.1	Good
AA-2	Spirilla serpens	1,077	30	35	0.7	Poor
Ad-13	Staphylococcus albus	597	30	19	0.0	Poor
AM-1	Phytomonas savastanoi	569	30	18	0.0	Poor
AM-3	P. tumefaciens	1,910	30	63	1.6	Good
AO-1	Cellulomonas biazotea	618	30	20	0.0	Poor
F-1	Actinobacillus lignieresi	645	30	21	0.0	Poor
G-17	Corynebacterium ovis	702	30	23	0.1	Poor
N-1	Micrococcus luteus	547	30	18	0.0	Poor
N-2	M. ureae	731	30	24	0.2	Poor
O-6	Mycobacterium phlei	779	30	25	0.2	Poor
O-15	M. smegmatis	803	30	26	0.3	Poor
O-21	M. berolinense	752	30	25	0.2	Poor
U	Pseudomonas ovalis	2,117	30	70	1.7	Good
U-6 (5 lines)*	Ps. fluorescens	1,043	43	24	0.2	Poor
U-6 (5 lines)†	Ps. fluorescens	2,462	41	60	1.5	Good

* 24-hour-old bacteria.
† 4-day-old bacteria.

DISCUSSION

The present findings indicate that among the food organisms selected only about one-third proved suitable for *P. multimicronucleata* cultured under these conditions. Since this investigation involved the use of a reproducible inorganic medium, one is hardly justified in comparing these results directly with those obtained by investigators using organic media such as various sterile hay or lettuce infusions, to which the bacterial inoculum was added. However, since disagreement already exists among other workers as to the suitability of the food organisms in the organic media, these results do afford a means of accounting for some of these discrepancies.

It has been shown in Table 1 that such species as *B. coli*, *B. subtilis*, and *Ps. fluorescens* have been reported as both suitable and unsuitable food organisms for various species of *Paramecium*. It has been shown here that, in the case of *E. coli*, strains K-11 and K-12 are quite different, the former being the better food. Such was also the case for *B. subtilis*, strain D-39 being far superior to strain D-40 as a food for the ciliate. Somewhat similar observations were made by Hetherington (1934) in his studies on *Colpidium*. He states:

> It is an interesting fact that *C. colpoda* is not very particular with regard to the species of bacterium upon which it will grow, although it may be more so than *C. campylum*. Indeed, the particular strain of the species used appears to be quite as important as the nature of the larger group. Thus the *coli* strain from Stanford (*Escherichia coli commune*) and *Aerobacter aerogenes* 5 gave trouble, while *E. communior* I and *A. aerogenes* from Stanford were as satisfactory as any bacterium tried.

Thus, upon strain differences within a given bacterial species the disagreement as to the suitability of a given food organism qualitatively standardized may well be explained.

It is also possible to account for the disagreement as to food qualities of a given bacterium in yet another way, since the suitability of *Ps. fluorescens* was found to vary according to the age of the bacteria. Since this species had been reported earlier as both suitable and unsuitable as food for *Paramecium*, strain U-6 of this bacterium was qualitatively standardized at 24 hours and 4 days of cultivation before use. It was found that at the latter age this bacterium served as an adequate control food for all the others tested, since over a period of 6 weeks 5 lines of the ciliate carried in it maintained a composite average daily fission-rate of 1.5. However, when this same strain of *Ps. fluorescens* was used after only 24 hours of cultivation, it proved to be a very unsatisfactory food. Of 5 lines of the ciliates started in the 1-day-old food only 3 were able to survive during the same period as the controls. Although cross-transfer of the paramecia was carried on, the composite average daily fission-rate of all lines started in this 1-day-old food was only 0.2. In comparison with the controls in the 4-day-old food, this very low division-rate indicates that the age of the food organism can be a deciding factor in determining the suitability of a specific food for *Paramecium*.

From this it should be pointed out that, since all the bacteria tested in this study were qualitatively standardized at 24 hours (except for the representatives of the Mycobacteriaceae), some of those which were found to be unsatisfactory may not prove to be so in older cultures.

The degree of suitability of the bacteria was roughly estimated by comparing the fed-animal cultures to one which was subcultured daily into sterile salt solution but without the addition of food. In unfed cultures the paramecia become greatly diminished in size and rather transparent prior to death, which occurs in about 12–14 days when the animal inoculum is 20 per cc. Giese and Taylor (1935) report that *P. multimicronucleata* will die of starvation in Peters' inorganic medium in about 12 days when there are 10 animals per cubic centimeter. Those food organisms which were found to be very unsatisfactory in some cases appeared to cause the death of successive cultures at almost regular intervals, which were much shorter than that required to kill the paramecia by starvation. For example, 7 successive cultures fed *A. lignieresi* died in 4 days of transfer.

In most cases the paramecia died in those foods which have been reported here as poor. However, in suspensions of *M. phlei*, *M. berolinense*, *M. smegmatis*, and *Corynebacterium ovis* the cultures never died out completely. In all these foods the paramecia ap-

peared much like those in the nonfed cultures, indicating that they were probably in a semistarved condition even though there was an abundance of bacteria present. Whether these members of the Mycobacteriaceae are only relatively "toxic" or are lacking in certain food requirements for the ciliate is a matter of conjecture in this ecological study. It is interesting to note, however, that *M. phlei* and *M. smegmatis* have been reported by D. F. Johnson (1936) as unfavorable foods for *Glaucoma ficaria*, cultured also in an inorganic medium. In those food suspensions which have been found here to be very satisfactory the paramecia flourished. This was especially true of those organisms fed *Ps. ovalis*, in which the fission-rate was 1.7. Giese and Taylor (1935) also found *Ps. ovalis* to be a suitable food for *P. multimicronucleata* cultured in a lettuce medium.

Recently Kidder and Stuart (1939) have studied the role of bacteria in the growth and reproduction of *Colpoda* grown in distilled water on 12 species of bacteria in pure culture. Their findings are somewhat similar to those reported here for paramecia. Their *Aerobacter cloacae* was most satisfactory, *Serratia marcescens* much less so, while *Staphylococcus albus* and *Ps. fluorescens* were decidedly unsuitable. Although the 3 species of *Pseudomonas* used were not identified other than by the numbers 81, 82, and 85, one is led to believe that these may be *Ps. fluorescens*. No attempts were made to standardize any of the food organisms.

These investigators did find that distilled-water culture medium will become a nutritive for many bacterial contaminants, as it becomes the solvent for ciliate metabolites and bacterial disintegration products. They found that by transferring the protozoa by means of a bacteriological loop every 48 hours into fresh medium over a period of 6 weeks bacterial contaminants (mostly *Pseudomonas*) so multiplied as to have a deleterious effect upon *Colpoda*.

In this investigation on paramecia such contamination was not observed. At the close of the 4-week period some of the protozoan cultures were tested for contamination. The final 1 cc. of used culture medium in each case was diluted with 9 cc. of sterile water, and these suspensions were examined by plating on nutrient agar. After a period of incubation these test plates showed only isolated colonies of the bacteria characteristic of the food organism used originally as the inoculum. This indicates that if any chance contaminant was present, it had not developed sufficiently to affect the results obtained. It is probable that because of the daily subculture methods the ciliate metabolites and bacterial disintegration products were not present in sufficient quantities to make the salt solution a nutrient for contaminating bacteria. Consequently, it is assumed that the paramecia were feeding on pure cultures of bacteria.

SUMMARY

1. Under standardized methods of culturing *P. multimicronucleata* this organism was found to utilize certain species of bacteria in pure culture as food organisms so that they maintained a high fission-rate over a relatively long period. Other bacteria proved to be less satisfactory foods, and still others were very unsatisfactory. In the latter case such foods caused the death of a given culture in a much shorter time than is usually required to kill a similar culture by starvation.

2. Strains within a given species of bacteria were shown to vary in food qualities as much as different species. This may account for differences previously reported in regard to the suitability of a given species as a food organism for the ciliate.

3. Aging *Ps. fluorescens* 4 days before use so improves its food qualities that it is a

very satisfactory food, whereas if this bacterium is taken from a 24-hour-old slant culture, it is definitely unsuitable.

4. The average daily fission-rate of the protozoa in a specific food suspension was used as a criterion of the suitability of the food organism. In those foods in which the average number of progeny of the ciliate was low this may be accounted for either by the dying-out of successive cultures or by the general unsuitability of the food. In either case the low average fission-rate represents rather clearly the degree of suitability of the food organism. Conversely, a high fission-rate indicates the superiority of the bacterium.

5. From the results of this investigation it is believed that the method of culturing paramecia in an inorganic medium on a pure strain of bacteria under standardized conditions will greatly facilitate experimental studies by insuring reproducible results. The determination of the suitability of various bacteria as possible food organisms under such standardized conditions as were employed should aid in the future selection of foods for *P. multimicronucleata* and should bring about closer agreement in future studies using the isolation-culture method.

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THE EFFECTS OF POPULATION DENSITY UPON GROWTH REPRODUCTION, AND SURVIVAL OF HYALELLA AZTECA

(Five figures)

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THE problems of the biotic relationships of like organisms have attracted much interest. Naturalists have reported with enthusiasm noteworthy aggregations, sexual behavior, and "social" manifestations. This work continues but with a more critical attitude. The less obvious mass relations, seen only by resultant effects, led to a dichotomy of interest, which in the future and to some extent at present (Park, 1939) will coalesce. One approach is that of the study of the whole population, its growth, maintenance, and decline or evolution (Chapman, 1928; Wright, 1931; Gause, 1934; Bodenheimer, 1938). The other is the study of physiological or behavioristic effects of population density upon the constituent organisms (Allee, 1931, 1934, 1938).

The physiological aspect is considered in the present study,[1] which attempts a demonstration of the effects of increasing the density of the population upon three fundamental processes: growth, reproduction, and survival. As an assay animal the common freshwater amphipod *Hyalella azteca* Saussure[2] (*H. knickerbockeri* Bate), Orchestiidae, was chosen. In the use of an aquatic arthropod the study is unique among researches comparable in nature to those of Pearl and of others to be cited later. The conditions of the experiments were variable, but equal volumes of food and water were used in all, irrespective of numbers of animals present. The following brief review of the literature is necessarily closely confined to the question studied and deals with recent studies on arthropods. The references given in the first paragraph include summaries of results on population studies of other forms. Earlier work by the same or different authors may be found by consultation of the literature referred to below.

Growth.—Body size (Warren, 1900) and rate of development (Banta, 1937) were retarded in Cladocera by crowding or its concomitant factors. Metamorphosis in the beetle *Tribolium confusum* was delayed, and body weight was reduced (Park, 1938) in relation to the density of the population and more directly to the degree of conditioning of the medium. On the other hand, evidence is present for an augmentative effect of increasing numbers of organisms. Development is accelerated in *Periplaneta orientalis* after the ninth instar, and the effect is apparent even in a density as low as 2 animals. However, the longest and heaviest animals were found among the isolated individuals, an effect attributed to increased disturbance by collision in higher densities (Landowski, 1938). Meal worms (*Tenebrio molitor*) raised 50 together in 60 cc. of meal consistently weighed more than did isolated larvae; even 150 animals in a similar volume maintained a higher weight than that of isolated ones until the sixtieth day (Michal, 1931). This was

I wish to express my gratitude to my adviser, Dr. W. C. Allee, for his interest in, and criticism of, this problem.

[2] To Mr. Clarence Shoemaker, assistant curator at the Smithsonian Institution, I am indebted for the identification of the animals.

attributed to an effect of increased temperature. Wardzinski (1938) believed this factor effective in the progressive acceleration of larval life in *Pieris brassicae* raised in densities ranging from 1 to 32 animals. The heaviest pupae of this butterfly occurred in populations of 2 or 4. Accelerated development in higher densities was found by Titschack (1936) and Mosebach-Pukowski (1937) in the clothes moth and *Vanessa*, respectively. Landowski (1938) cites Manujlowa, Kosmina, and Alpatov (1931), and Alpatov and Dorodnichaia (1932), as obtaining results indicating the inhibitory action of isolation on lepidopterans.

Reproduction.—Few studies on reproduction in regard to density in arthropods are at hand. The most complete concern *Drosophila melanogaster*, which showed a clearly inverse relationship to numbers of animals present (Pearl, 1932). In cladocerans reproduction was adversely affected by crowding (von Dehn, 1937). The retardative effect of crowding on the group fecundity of scales (*Lepidosaphes ulmi*) was due to the increased number of sterile females rather than to an effect upon the reproducing mothers (Smirnov and Polejaeff, 1934). On the other hand, crowding decreased fecundity in the moths *Arctia caja* and *Lymantria dispar* by lowering the maturation of ovarian eggs. This effect was attributed to malnutrition through disturbance by collision during feeding (Hofmann, 1933).

By counts of the growing populations the reproductive activity of flour beetles seems to be maximal at a population of intermediate density (Park, 1932; Maclagen, 1932; Maclagen and Dunn, 1936). Increased copulation frequency was directly shown to increase fecundity of *Tribolium* (Park, 1933) and may be operative in the greater population development of an initially intermediate density. However, this effect is offset to an increasing extent by the direct retardation of fecundity by increased conditioning of the medium (Park and Woollcott, 1937).

Survival.—A rough generalization on the effects of density upon survival may be attempted: under toxic conditions or those inimical by the absence of essential elements, survival is maximal at an intermediate density since the organisms or their products tend to ameliorate these conditions. Many instances are given by Allee (1931) and, more recently, by Allee and Wilder (1939). Careful analysis of death rate in *Drosophila* populations (Pearl, Miner, and Parker, 1927) demonstrated a similar effect, with the optimal density at 25–35 flies per 1-ounce bottle. However, survival is frequently inverse to the numbers of organisms present. This has been reported for cockroaches (Landowski, 1938) and for larval mortality of grain beetles (Maclagen and Dunn, 1936; Park, 1938).

MATERIAL AND METHODS

Hyalella azteca Saussure (*H. knickerbockeri* Bate) is a widely distributed amphipod. The specimens which were used came from a culture maintained for over ten years in the tanks of the department greenhouse, where they were accidentally seeded.

None of the research done upon amphipods has required the development of the standardized techniques necessary in quantitative studies on physiological effects. The most complete study of amphipods is that initiated by Sexton and continued by herself and others. This chiefly genetic study has been summarized by Sexton and Clark (1936). In their work the animals were kept in finger bowls and fed upon *Ulva* or dead leaves—a method altogether unsatisfactory for results dependent on the control of all factors, as far as possible, other than density of like organisms. In the search for greater control

much of the present work is exploratory and covers a rather wide field of observation in order to lay a basis for further analytical treatment.

The experimental procedure consisted in obtaining the needed number of newly released young within, if possible, 2–3 days. They were then counted out at random into 6- or 8-ounce, narrow-necked bottles, according to the planned population densities. The bottles had been filled with 100 cc. of water, to which were added either 2-inch lengths of *Elodea* or 2–4 drops of yeast. Usually the food and water was changed weekly until the end of the experiment. All experiments were conducted at room temperature, which was recorded by a thermograph (Table 1). Temperatures are given in degrees centigrade.

The above résumé needs further expansion. Several hundred females with brood in about the same stage were collected from the stock and kept in finger bowls. Shortly before the time of expected release of the young, the bowls were cleaned of all young then present; from that time on they were removed at frequent intervals and stored together in finger bowls for use in the experiment.

Elodea was first tried as food because it is one of the natural food plants of the amphipods (Embody, 1912). The long streamers were washed, stripped of their leaves, and scalded in hot tap water. The latter treatment was planned to kill associated organisms. The food was most unsatisfactory because of inequality along the length of the stem and from stem to stem. The hot-water treatment sometimes killed the plant and did not always kill the animals on the plant. For these reasons *Elodea* was abandoned after the third experiment.

Yeast was substituted, since it is a simple, easily measured food. Fleischman's yeast cake was made up with distilled water in the proportion of 1 gm. to 10 cc. The well-stirred stock suspension was fed to the populations by drop. The yeast in the population bottles was not itself growing but started to grow upon removal to a suitable nutrient solution. This took place even after the yeast had been 8 days in the clean water medium. No attempt at sterile precautions or at bacterial analyses has been made.

The initial densities seeded changed with the deaths of some of the animals. In order to maintain, as nearly as possible, the original densities, one or more bottles of a given population were eliminated, and the animals used to restore the numbers in the remaining bottles of that density. This could not be done in densities of 20, 50, and 100, where few bottles were available, especially in the two highest densities. Here the densities were allowed to drop but never to overlap. These populations were equalized by transference of animals between bottles of a given density.

The molted skins (exuviae) were collected at each time of change of water, or oftener, and preserved in 50 per cent alcohol. Later, magnified projection outlines of the mid-dorsal lines were drawn, and these were measured by calipers.[3] It is necessary to use the exuviae for an accurate total-length measure because the animals are flexible, and the segments telescope within each other. The living whole animals, even when anesthetized, displayed all degrees of flexion. Since the exuviae were transparent, the overlap of segments might be seen, and the drawings were made to include this factor. The total lengths given, therefore, are those of the summated sclerites. The figures are the most accurate possible, but they are entirely dependent on a rough sampling of the population. As the animals became older, the validity of the representation suffered greatly because they molted less often, and they destroyed the exuviae. The latter was particularly

[3] I wish to thank Miss Catherine Lutherman for her assistance in these measurements.

TABLE 1

EXPERIMENTAL CONDITIONS

Expt. Number	Initial Density	Number of Bottles	Remarks
Elodea I.....	2	25	Started 12/7/37, mean age of young: 2 days; temperature: mean 19°.3, range 16°.0–23°.0; food and water: *Elodea*, tap water, both changed every other week; total number animals seeded: 450
	5	10	
	10	5	
	25	4	
	50	2	
	100	1	
Elodea II.....	1	50	Started 1/16/38, mean age of young: 2 days; temperature: mean 19°.4, range 16°.0–24°.0; food and water: *Elodea* (living), charcoal-filtered tap water changed weekly; total number animals seeded: 190
	10	5	
	20	2	
	50	1	
Elodea III....	1	50	Started 1/16/38, mean age of young: 6 days; temperature: mean 19°.4, range 16°.0–24°.0; food and water: *Elodea* (living), charcoal-filtered tap water, never changed, poured through silk bolting cloth weekly; total number animals seeded: 180
	8	5	
	20	2	
	50	1	
Yeast I.....	1	50	Started 5/3/38, mean age of young: 1 day; temperature: mean 25°.2, range 21°.0–30°.0; food and water: 2 drops yeast to 6/14, then 4 drops, filtered tap water, both changed weekly; total number animals seeded: 750
	2	50	
	4	25	
	10	20	
	20	5	
	50	2	
	100	1	
Yeast II.....	1	100	Started 8/3/38, mean age of young: 2 days; temperature: mean 28°.0, range 24°.0–31°.3; food and water: 4 drops of yeast, filtered tap water, both changed weekly; total number animals seeded: 820
	2	50	
	4	25	
	6	20	
	10	20	
	20	10	
Yeast III....	1	100	Started 12/7/39, mean age of young: 2 days; temperature: mean 21°.0, range 19°.5–22°.0; food and water: 4 drops yeast, filtered tap water, both changed weekly; chloretone used after second week; total number animals seeded: 640; replacements on fourth day
	2	60	
	4	25	
	6	20	
	10	10	
	20	5	
Yeast IV.....	1	60	Started 2/22/39, mean age of young: 4 days; temperature: mean 20°.9, range 19°.5–22°.0; food and water: 4 drops yeast, well water, both changed weekly; chloretone used after second week; total number animals seeded: 420
	2	30	
	4	15	
	6	10	
	10	6	
	20	3	
Yeast V.....	1	100	Started 2/26/39, mean age of young: 3 days; temperature: mean 20°.9, range 18°.6–24°.0; food and water: 4 drops yeast, supplemented after 1 month of age by 2 inches *Elodea* stem, well water, both changed weekly; chloretone used after one month; total number animals seeded: 1,020
	2	50	
	4	25	
	6	20	
	10	20	
	20	10	
	50	4	
Yeast VI.....	1	80	Started 5/10/39, mean age of young: 4 days; temperature: mean 24°.9, range 19°.2–30°.0; food and water: 4 drops yeast, well water, both changed weekly; chloretone used in first week; total number animals seeded: 564
	2	40	
	4	20	
	6	14	
	10	8	
	20	4	

true in a density of 10 or more. The measurement of size by exuviae has been used in a study of proportional growth in *Gammarus* (Sexton, 1924).

A second method was used after experiment Yeast II. All animals of a population were anesthetized in chloretone, and the head length was measured by ocular microm eter. The increased deaths from this method invalidated the survival record. However, the error in the growth analysis due to partial sampling was entirely obviated. To substantiate the results obtained in growth by measurements of body or head lengths the sum of the antennal segments of the first and second antennae of one side was usually recorded also ("antennal segment number").

Fecundity was demonstrated by removal and preservation of the females carrying brood. They were later measured, and the eggs stripped from the pouch and counted. Only three experiments lasted long enough with a sufficiently high survival to provide such data.

By counting the animals when the water was changed, the number surviving was easily obtained. Survival has been followed only until the time of female maturity, to avoid the necessity of a correction factor for the removed females. Also survival records have not been used after anesthesia by chloretone.

EXPERIMENTAL RESULTS

GROWTH

Isolated controls.—The broods of 10 females were isolated, on May 4, 1938, 1 young per 100 cc. of filtered lake water with 1 drop of yeast. Initially, 107 first-instar animals were obtained. The water and food was changed weekly; at the thirty-eighth day the

TABLE 2*

GROWTH OF ISOLATED *H. azteca* FED UPON YEAST FROM THE FIRST TO THE TENTH INSTAR

Instar	Days of Age at Given Molt	Duration of Instar (Days)	Body Length (Mm.)	Increments of Growth (Mm.)	Percentage Head to Total Length	Number of Antennal Segments of One Side
1.....	5.2±0.1	5.2±0.1	1.28±0.01	.....	13.6	13.0±0.1
2.....	10.7±0.2	5.4±0.3	1.53±0.02	0.24±0.02	12.5	13.6±0.1
3.....	16.6±0.3	5.9±0.2	1.83±0.03	0.31±0.02	12.0	14.4±0.2
4.....	22.6±0.4	6.1±0.3	2.17±0.04	0.33±0.03	11.2	15.2±0.2
5.....	28.9±0.4	6.1±0.3	2.48±0.05	0.31±0.03	10.9	16.6±0.2
6.....	34.5±0.6	5.9±0.3	2.83±0.04	0.35±0.02	10.4	17.6±0.2
7.....	41.9±1.1	7.6±0.3	3.23±0.06	0.38±0.02	10.1	18.9±0.3
8.....	49.6±1.2	8.8±0.4	3.65±0.06	0.42±0.02	10.0	19.9±0.3
9.....	58.0±0.7	9.3±0.4	4.00±0.08	0.38±0.03	9.5	21.1±0.3
10.....	65.2±1.2	9.1±0.4	4.40±0.11	0.37±0.04	9.4	22.1±0.7

* Means and standard errors are calculated from 72 animals providing data on time and age, of which 42 were used in the calculation of morphological values.

food was increased to 2 drops of yeast, and on the seventy-second day to 4 drops. This experiment was run synchronously with Yeast I; therefore the conditions are comparable to those of that experiment. The bottles were observed daily, molts recorded, and exuviae preserved for later measurement. Only the data pertinent to this problem will be discussed. Deaths reduced the number of animals to 73, and of these only 42 series of exuviae were sufficiently complete for growth records. Table 2 presents the mean values and their standard errors.

TABLE 3

MEAN BODY LENGTH (MM.) OF ANIMALS IN POPULATIONS FED UPON *Elodea*

(The lower half of each table presents the significance [*P*] of the difference between the designated populations)

A. *Elodea* I

Age in Days	Initial Population Density					
	2	5	10	25	50	100
16	2.02	2.10	2.19	2.18	2.11	1.84
40	4.27	3.75	3.79	3.83	3.33	2.51
45	5.32	4.70	4.48	4.25	3.72	2.90
52	5.79	5.64	5.41	4.47	3.78	2.95

Age in Days	Compared Population Densities					
	2:5	5:10	10:25	25:50	50:100	2:10
16	0.44*	0.33	0.84	0.16	0.008	0.11
40	0.04	0.22	0.02	0.001	0.0001	.....
45	0.03	0.38	0.27	0.02	0.002	.....
52	0.63	0.49	0.01	0.06	0.010	.....

B. *Elodea* II

Age in Days	Initial Population Density			
	1	10	20	50
9	1.39	1.38	1.34	1.23
16	1.91	1.76	1.89	1.72
23	2.32	2.44	2.25	1.87
30	3.16	2.97	.....	.....
72†	5.18	4.27	3.69	3.04

Age in Days	Compared Population Densities		
	1:10	10:20	20:50
9	0.92	0.42	0.11
16	0.13	0.13	0.06
23	0.62	0.39	0.12
30	0.43	.....	.....
72	0.0001	0.09	0.15

* The values are probabilities (*P*). A *P* of 0.05 (5 chances in 100 of getting a similar difference by "chance") is regarded as the upper limit of significance. The smaller the decimal the greater is the significance.

† Lengths are of all survivors. Sexes approximately equally represented.

The changes from instar to instar were quantitative; no qualitative criteria were found. The antennal segment number for each instar was highly variable, and there are indications that it cannot be regarded as a valid index of instar. Sexual differentiation also was gradual. No earlier than the seventh instar (about 40 days), often much later, the males might be distinguished by the heterogonic development of the large second gnathopods. On the average, the males in the experiments were larger after maturity than were the females. In the experimental resuits presented later the percentage of developed males is given, when possible, to indicate whether disproportion of the sexes among the populations is correlated with mean size differential. The first molt occurred at about 5 days of age (Table 2). In the experiments body length was first measured at least 1 week after the start of the experiment, at which time the animals would usually be between 8 and 11 days of age. Therefore, the first recorded mean size covers a mixture of first -and second-instar animals. Table 2 shows also the percentage relation of head to total length, since these two measures have been utilized in the experimental results.

TABLE 4*

GROWTH IN POPULATIONS FED UPON YEAST IN EXPERIMENTS I–VI

Age in Days	Initial Population Density					
	1	2	4	6	10	20
10.4	1.67	1.73	1.76	**1.78**	1.77	**1.78**
17.6	2.41	2.47	2.55	2.52	2.43	**2.56**
26.2	2.96	3.00	**3.02**	2.98	2.84	2.84
33.7	3.83	**3.96**	3.89	3.56	3.44	3.32
44.0	**4.51**	4.29	4.41	4.23	4.13	3.95

* The values are means of total length and head length ×10 (mm.). The boldface figures are maximal for each age.

Growth of animals in populations fed on Elodea.—Of the three experiments in which *Elodea* alone was used as food, only two furnished data upon the growth of the animals. In the third experiment, in which the water was never changed, insufficient numbers of molted skins were obtained, either because the animals molted rarely or at once destroyed them. From Table 3 it may be seen that, on the whole, mean body size was inversely related to the density of the population. The maximal size at density 10—16 days—in *Elodea* I was not significant, but the indication is of interest in view of later results. The significance of the difference between densities increased with age (Table 3, *A*, 16 days compared to 40 days) and with increasing density (25:50, compared to 50:100). In *Elodea* II none of the observed differences is significant until age 72 days, at which time the isolated individuals were significantly larger than those in population 10.

Growth of animals in populations fed upon yeast.—When compared to the results of *Elodea* feeding, animals in populations fed upon yeast show another effect. Table 4 presents an average of the six experiments at the ages and densities which are comparable. Population density 6 has been interpolated for Yeast I. The results on head length in Yeast III through VI have been multiplied by 10 to weight them nearly equal to body lengths. The table shows a shift of mean maximum size from a high density to lower den-

sities with advancing age. The results of separate experiments bring this point out more clearly. Figure 1 presents the results of the first yeast experiment. In this experiment only 2 drops of the stock yeast suspension were added to the 100 cc. of filtered lake water. After the experiment was in progress, it became apparent that this was not sufficient, since population 10 cleaned it out of the water before the week between feedings was complete. However, such feeding was continued until the maximal growth of population 1 was well established (Fig. 1, 43 days). On the forty-fifth day bi-weekly feeding was started, making a total of 4 drops per week to each population, an amount used in all later experiments. Only the densities through 20 have been used in the figure. Density 100 provided practically no exuviae for length measurements; the density of 50 and 20 gave data through the twenty-fifth day. The failure of data for these higher densities is largely due to the destruction of the exuviae and, possibly, to less frequent molting. Consequently, the few molted skins obtained were an unreliable sample. However, the trend of the higher densities is indicated at the eighty-eighth day by the result in density 10 and 20. At this age all remaining males were preserved and later measured so that the values are derived from the whole male population. The populations did not invariably fit the trend. Pearl and Parker (1922) write that, owing to accidents relative to the smallness of numbers at certain populations, their populations did not "fall in line." The discussion of survival will show this more clearly in the amphipod populations (Fig. 5).

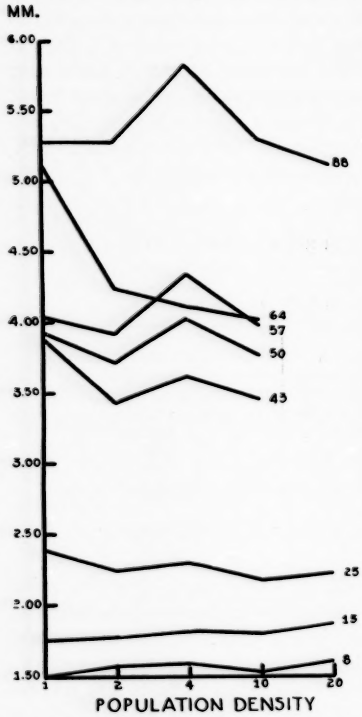

FIG. 1.—Mean body length of populations in Yeast I. Age in days to the right of each curve.

During the first 2 weeks of age the animals of population 20 were significantly larger than those isolated ($P = 0.017$ and 0.002 for 8 and 15 days of age). Thereafter, body length tended to become inverse to the population density, although the difference between densities of 1 and 2 was not significant. After the food was doubled in quantity, an insignificant ($P = 0.271$) maximum occurred at density 4 through the fifty-seventh day. Failure of significance is related to the small number of exuviae obtained in any population and their great variability in length. At the sixty-fourth day the isolated animals were on the average of greatest body length. The difference between densities 1 and 4 is significant ($P = 0.028$), despite the fact that the 24 isolated animals for that day were represented by 6 exuviae only. The sampled animals of population 4 were of smaller size in general than on the preceding date (57 days). Probably those molted skins were from females, which at sexual maturity show the inverse relationship of body length to density (Fig. 4, *C*) and which at that time, in general, are smaller than the males. Males, on the

other hand, in the three experiments (Yeast I, IV, V) which continued long enough for this measure, invariably were of greatest mean length in a density of 4 (Fig. 3). The latter observation was shown in Yeast I by the mean body lengths of the preserved males at the eighty-eighth day of age. This curve strongly suggests those of the three ages prior to the sixty-fourth day. Counts of the developed males made on the sixty-eighth day showed the highest proportion of that sex in the population density of 4; the percentages for populations 1–50 were, respectively, 41.2, 54.0, 64.9, 51.2, 48.0, and 14.6.

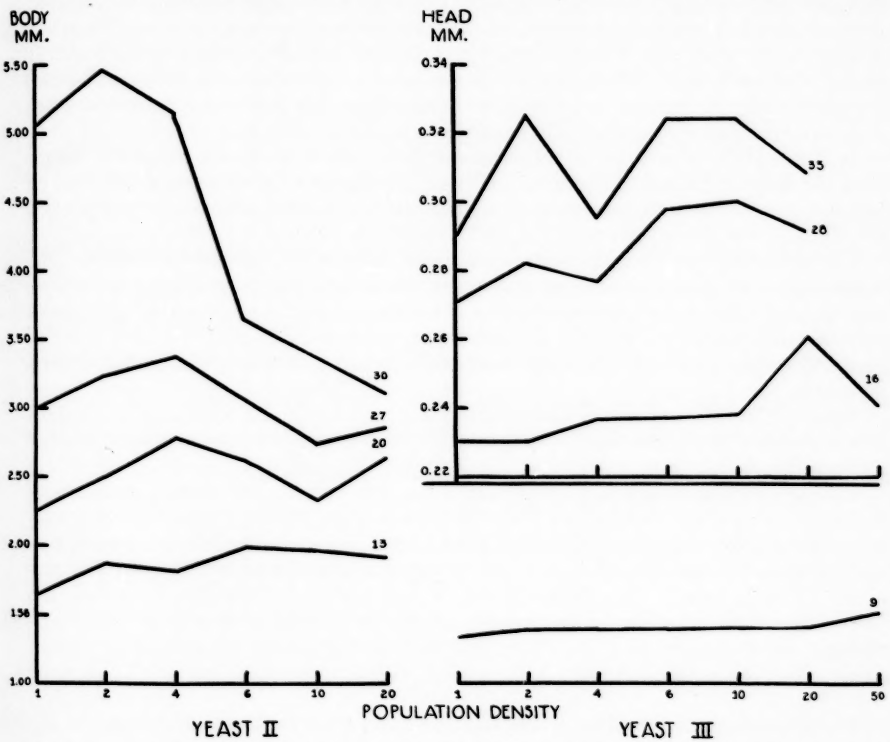

FIG. 2.—Mean body or head length of populations fed upon yeast. Age in days to the right of each curve

These figures are not basic sex ratios, which are approximately 1:1, because the females which had shed their eggs had been removed from the population and because the immature males are practically indistinguishable from females without brood or mature ovaries. There is, unquestionably, delay of sexual maturity in populations of 50 or more. The percentages given above indicate that the preponderance of developed males may have occasioned the peak of size at population 4 from the forty-third day.

The next two experiments were planned to demonstrate the growth of the animals in the populations prior to sexual maturity, in order to obviate the complication introduced by the presence of mature males and females. Both are presented in Figure 2. The sec-

ond type of length measure was instituted in the second week of Yeast III (16 days of age); all animals were anesthetized, and the head lengths measured by an ocular micrometer. The values are, therefore, means of the entire population. The same is true of the thirtieth day in Yeast II, for the values are derived from measures of the whole preserved animals. The results show a shift of mean maximal size from a higher density to a lower with advancing age. In Yeast I (Fig. 1) the isolated animals were maximal in size on the twenty-fifth day; with double the food the shift in Yeast II and Yeast III had fallen to the density of 2 at the thirtieth and thirty-fifth days, respectively. The delayed drop of the maximum may be correlated with the increased quantity of food. Yeast III started on the ninth day with maximal exuvial length in population 50, but in Yeast II on the thirteenth day the population 6 is the largest. The difference between the two experiments is not explainable by changes in technique, but it does not seem unreasonable, since the former was started in December and the latter in August.

In Yeast II the population of mean maximal size was at no time significantly bigger than the isolated animals. However, in Yeast III significance obtained at all but the last age; the *P* values, the maximum compared with the isolated animals at the four ages, are, respectively, 0.003, 0.0001, 0.016, and 0.093.

The preceding three experiments have given reasonably comparable results. The water used in all three was charcoal-filtered Lake Michigan (city) water from the laboratory tap. In order to see whether the drift of maximal growth was related to the type of water used, a change was made in the next three experiments (Yeast IV, V, VI) to Whitman well water. Quantity of food was maintained as in Yeast II and Yeast III at 4 drops per week.

Yeast IV has been chosen to be presented in complete tabular form (Table 5), since this experiment included exuvial length, head length, and antennal segment number through the sixtieth day.

The outstanding feature of Yeast II and Yeast III—namely, the shift of mean maximal length from a high density to a lower—occurred again, although somewhat less regularly. With great individual variation, as has been previously mentioned, sexual maturity starts after the fifth week, so that the maximal size of density 4, from the thirty-ninth day, as in Yeast I, may be influenced by the presence of the males. As before, the percentage of developed males was greatest among the animals of density 4. These percentages are 41.6, 35.7, 46.1, 34.8, 41.2, and 35.0 for population densities 1–20, respectively. Also the males of density 4 are largest, although not significantly (Table 5, 60 days; Fig. 3).

The absence of exuvial values for populations 2 and 10 on the thirty-second day of age is due to the fact that no molted skins represented those populations. The destruction of "molts" throughout all populations was so great thereafter that collection of them was abandoned. The data on head length, by their freedom from sampling error, are more reliable, but both measurements show in part a significantly greater size of the animals in the higher densities at an early age. The animals represented on a given date by molts were of an earlier instar than the same animals within the whole population valued by head length on the same date. In *C* with exceptions the number of antennal segments tends to support the growth effect presented in *A* on the basis of length.

Yeast V was designed originally for the study of survival and fecundity only. It was very difficult to see the eggs within the brood pouch in yeast-fed females because the yolk remains practically colorless. The addition of *Elodea* to the 4 drops of yeast after

TABLE 5

GROWTH OF ANIMALS IN YEAST IV

A. MEAN BODY (*a*) AND HEAD (*b*) LENGTHS IN MILLIMETERS

AGE IN DAYS	INITIAL POPULATION DENSITY					
	1	2	4	6	10	20
11 a*	1.56	1.50	**1.67**	1.65	**1.67**	1.66
18 a	1.96	1.93	1.97	1.84	1.86	**2.04**
b†	0.27	0.27	**0.28**	**0.28**	**0.28**	**0.28**
25 a	2.43	2.45	**2.54**	2.43	2.42	2.43
b	0.31	0.31	**0.32**	0.31	0.30	0.31
32 a	2.98	.....	**3.26**	2.89	.....	2.90
b	0.36	**0.38**	0.37	0.35	0.34	0.34
39 b	0.40	0.41	**0.43**	0.39	0.39	0.38
46 b	0.45	0.44	**0.46**	0.43	0.44	0.40
53 b	**0.48**	0.47	**0.48**	0.45	0.47	0.46
60 b‡	0.51	0.52	**0.53**	0.52	0.51	0.46

B. SIGNIFICANCE (*P*) OF DIFFERENCE BETWEEN ABOVE MEAN LENGTHS

AGE IN DAYS	COMPARED POPULATION DENSITIES								
	1:2	2:4	4:6	6:10	10:20	1:4	1:6	1:10	1:20
11 a	0.42	**0.016**	0.37	0.62	0.92	**0.004**	**0.021**	**0.021**	**0.004**
18 a	0.69	0.37	**0.004**	0.48	**0.009**	0.84	0.09	0.23	0.33
b	0.84	0.09	0.92	0.32	0.37	.....	**0.02**	.....	**0.036**
25 a	0.84	0.48	0.19	0.76	0.92	0.32	.....	.....	.....
b	.....	0.48	0.19	0.13	0.11	0.48	.....	.....	.....
32 a	.....	.....	0.13	.....	.....	0.23	.....	.....	.....
b	**0.028**	0.62	0.06	0.27	0.55	.....	.....	.....	.....
39 b	0.48	0.31	**0.009**	0.48	0.32	0.09	.....	.....	.....
46 b	0.84	0.42	0.07	0.32	**0.0001**	0.61	.....	.....	.....
53 b	0.56	0.55	0.19	0.48	0.84	.....	.....	.....	.....
60 b	0.62	0.84	0.69	0.42	**0.001**	0.54	.....	.....	.....

C. ANTENNAL SEGMENT NUMBERS

AGE IN DAYS	INITIAL POPULATION DENSITY					
	1	2	4	6	10	20
11 a*	13.0	13.0	13.0	13.0	13.0	**13.1**
18 a	**14.8**	14.3	14.6	14.6	14.6	**14.8**
b†	15.7	15.4	**15.9**	15.7	15.5	15.7
25 a	15.7	16.1	**16.3**	16.2	15.4	**16.3**
b	16.9	17.1	**17.2**	17.0	17.0	**17.2**
32 a	**19.0**	.....	18.6	16.8	.....	17.6
b	19.3	**19.5**	19.4	18.4	18.3	17.9
39 b	20.4	21.1	**21.2**	20.4	19.8	19.4
46 b	22.2	22.1	22.2	**22.7**	21.3	20.3
53 b	**23.4**	22.7	22.8	21.9	22.1	21.1
60 b‡	23.8	23.2	**24.4**	22.3	22.8	21.6

* *a* = body length or antennal segment number derived from exuviae.
† *b* = head length or antennal segment number from anesthetized animals, except at 60 days when preserved males used.
‡ Each population represented only by males.

the first month was planned to obtain green-colored eggs. It was hoped that by starting a large experiment, not using choloretone, and feeding in this way, a great number of fecund females would be obtained. After 1 month of age the survival was so high that it was decided to risk the deaths by chloretone and take a measure of growth. The readings after the first were of animals fed yeast and *Elodea*.

At the age of 34 days the animals in density 4 were of significantly ($P = 0.028$) longer average head length (0.38 mm.) than those in density 2 (0.36 mm.), which did not differ significantly ($P = 0.76$) from isolated animals (0.36 mm.). The higher populations showed a drop progressively, starting with 6 (0.36 mm.). At 48 days of age, 14 days after the first *Elodea* feeding, the isolated animals were of insignificantly maximal size ($P = 0.33$ when compared with density 2). As before, the males of density 4, on the sixty-second and seventy-sixth day were maximal in head length and antennal number

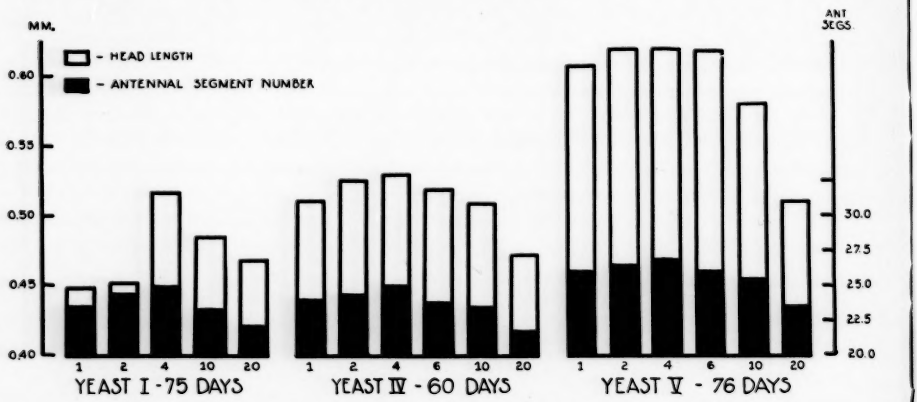

FIG. 3.—Head lengths (left ordinates) and antennal segment numbers (right ordinates) of sexually mature males fed upon yeast. Abscissae, population density.

(Fig. 3). This difference was not significant ($P = 0.19$). The results on mature males in Yeast I, IV, and V are presented in Figure 3. The head lengths of these males are not significantly greater than those of sparser populations except in Yeast I, where the mean head size of density 4 has a P value of 0.007 over that of density 2. The body length of sexually mature females, on the other hand, is inverse to density at the mean age of first oviposition (Fig. 4, *C*).

Yeast VI was started at the same time as Yeast V and was designed purely to recheck the growth effect. Since no survival data were required, chloretone anesthesia was begun at the end of the first week (11 days of age). Either for this reason or for some other, the results were atypical in comparison with the other yeast experiments. At 11 days of age maximal head size was obtained in the density of 4, but thereafter the mean greatest head length was displayed by 2, 1 and 2, 2, and 4 in that order in the five determinations covering the 39 days of the experiment. Neither do the antennal segment numbers correlate well with head size. None of the maxima is significantly greater than the mean value from sparser populations.

When measurements were made on the eleventh day one-half of the animals (40)

were anesthetized and measured. It would have been impossible to measure 80 animals in 6 densities within one day, hence the number was halved. This may have contributed to the atypical results, but two technically interesting facts emerged. The deaths were much greater the week after the use of chloretone on those animals, 18.1 per cent as compared to 8.0 ($P = 0.001$), and they were of smaller mean head size; the difference of 0.015 mm. is significant ($P = 0.0004$).

In conclusion, from the results on growth in relation to the density of the population two effects have emerged. When the animals were fed upon *Elodea*, growth was inversely related to the density of the population in city water with and without chlorine. In the former case a slight indication of a maximum in a density of 10 was obtained at the sixteenth day, but this was not significant. However, when yeast was used as food and the water was either dechlorinated city water or well water, early growth was greatest at a high density; but with increasing age animals in lower densities were, on the average, larger. This was a fairly regular shift. The above trend recurred in five experiments, and frequently a statistically significant difference was observed. This was especially true when the compared densities were several steps apart in the experimental gradation. There is an indication that increased quantity of yeast will shift the maximum toward higher densities. The mature males of mean greatest size occurred without exception in the population density of 4. The isolated mature females, on the other hand, at the average age of shedding of first eggs were of largest size.

REPRODUCTION

Behavior.—A mature female oviposits about every 8 days. The eggs are incubated for about 6 days, and shortly thereafter the hatched young leave the pouch. Toward the end of incubation a male carries the female by hooking the dactyl of the first (small) gnathopods under the lateral edges of the second thoracic segment. Amplexus may last only 1 day prior to oviposition, or it may continue much longer if the female is skipping one or more ovulations; a duration of 28 days continuous amplexus has been observed. However, it apparently never occurs during the first 3 days of incubation. On the average it may be said that the male carries the female for about 4 days. During this time release of the brood of the previous mating, molting of the female, insemination, and oviposition occur.

In *H. azteca* the male does not necessarily desert the female at molting. In fact, he may apparently assist the molt (Sexton and Matthews, 1913) by arching up over the female's back and scraping the integument with the uropods. Afterward, insemination occurs during a frequently repeated palpation of the base of the pouch. During this action the male shifts his grasp on the female in order to lie diagonally crosswise and to curve his abdomen beneath that of the female. Oviposition usually takes place within 12 hours of molting, and further attempts at copulation are vigorously resisted, although the male continues for some hours to carry the female.

Female amphipods of many kinds are said never to shed eggs unless carried at some time in the cycle by a male. Embody (1912) cites this to be the case for all amphipods which he studied, including *H. knickerbockeri* (a synonym of *H. azteca*). However, in the present experiments females isolated from the first instar shed eggs. Sexton (1924) reports failure of shedding eggs by isolated females of *Gammarus chevreuxi* and *G. pulex*, but not in *G. locusta*. There were, however, indications of a larger brood size in the latter females when mated. The sterile eggs do not develop and are cast within a few days.

While compiling data upon life-history, counts of the broods of 346 stock females were made at various times. On the average 5.2 eggs were produced per stock female. Two tests of the correlation between brood size and female size (using head length as an index) were made. Forty-six animals measured on July 12, 1937, showed an average head length of 0.510 mm. and a brood size of 4.5 eggs. The correlation is significant ($r = 0.627 \pm 0.089$).[4] Seventy-four others (July, 1939) gave 0.493 mm. head length and 4.0 eggs per brood ($r = 0.537 \pm 0.083$). Since amphipods increase in size at each molt, even after sexual maturity, it is impossible to state with these measurements whether the correlation is more directly related to female size or to female age.

An increase in fecundity with increasing age, presumably up to a certain limit, has been reported for amphipods (O'Brien and Yarnold, 1937; Embody, 1912; Sexton and Matthews, 1913). However, these authors did not attempt to segregate the factors of age and body size.

The effect of density.—Two hundred and sixty-eight females from three experiments provided the data to be discussed and represent three conditions of food: *Elodea* (Elodea I); yeast supplemented after 1 month by *Elodea* (Yeast V); and yeast alone (Yeast I). The results are graphically presented in Figure 4. In *Elodea* I densities 5 and 25 have been plotted with 4 and 20. Density 100 is omitted from the curve of *Elodea* I because at the time of last observation (70 days) only 2 females had shed eggs. Omitted also are densities 100 and 50 from Yeast I, since no matured females represented the first and only one the second at the seventy-seventh or last day of record. Otherwise, all densities of the three experiments are represented by from 5 to 30 females. The higher numbers occurred in densities 10–25, probably correlated with the higher survival (Fig. 5). The lack of females from densities 100 and 50, however, was not associated with a low survival but resulted from an actual retardation or inhibition of maturity. This is presaged by the retarded age at sexual maturity of the animals in density 20 and also in density 50 where represented (Fig. 4, *B*).

By analysis of the combined data from populations 2, 4, 10, and 20, which are densities present in all three experiments, a few observations pertinent to the relation of reproduction and diet may be drawn. The differences mentioned below are highly significant ($P < 0.00001$), unless otherwise stated. The sexually active females fed upon *Elodea* throughout life were larger (5.62 mm.) than those fed upon yeast for all (4.56 mm.) or for the first month of life (4.47 mm.). The addition of *Elodea* after 1 month had no significant effect on body size when these animals are compared with those fed upon yeast continually ($P = 0.19$).

The fecundity of the *Elodea*-fed females (8.9 eggs per brood) was much higher than that of the yeast-fed (4.7 eggs per brood). The addition of *Elodea* to a yeast diet significantly increased fecundity (5.9 eggs, $P = 0.005$), but the fecundity from the constant *Elodea* diet was even higher ($P = 0.0001$).

As the yeast-fed animals were retarded in body length and fecundity, so also were they retarded in age at the shedding of the first eggs (60.7 days old) compared to that of the *Elodea*-fed animals (52.9 days). The most rapid development occurred, however, among the animals fed upon both (47.7 days).

In such comparisons it is necessary to consider temperature. The maximal response of the *Elodea*-fed females occurred in a midwinter experiment (19°.3 average temperature), the retarded yeast-fed animals were living under a mean temperature of 25°.2 in

[4] An r value which is greater than twice its standard error has statistical significance.

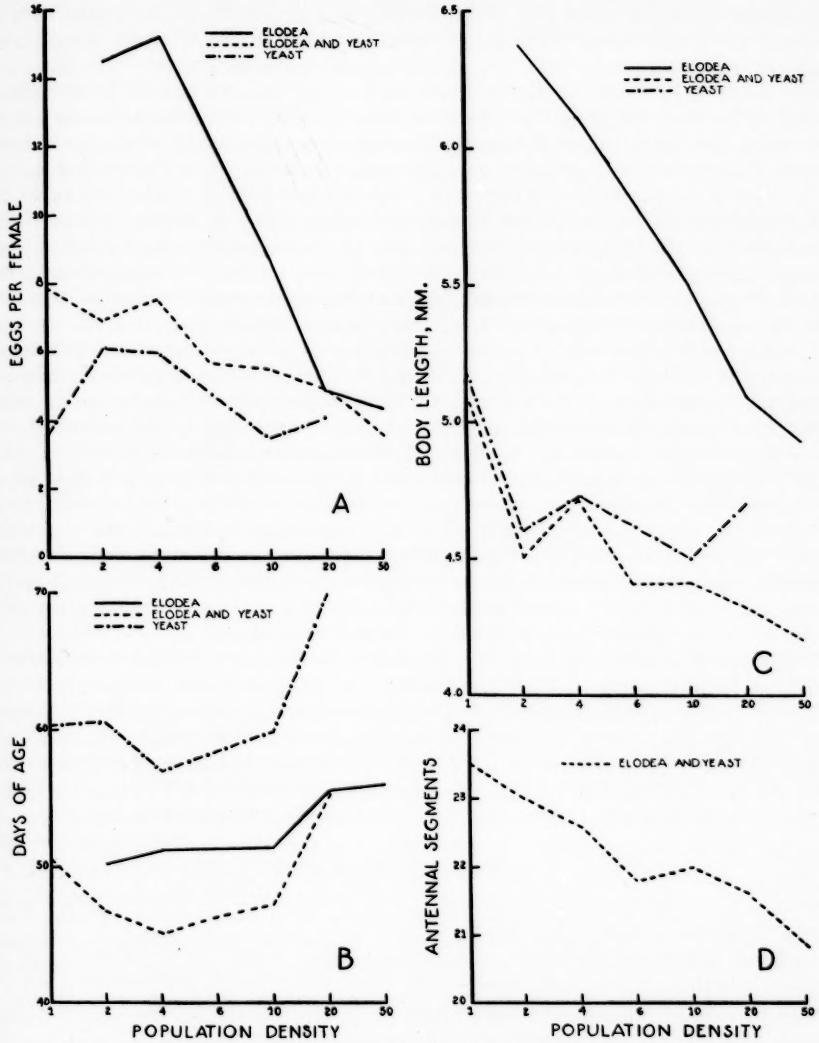

FIG. 4.—Mean fecundity (*A*), age at first oviposition (*B*), body length (*C*), and antennal segment numbers (*D*) of females in Elodea I (———), Yeast V (– – –), and Yeast I (— · —).

midsummer, and the yeast and *Elodea* feeding was conducted in the spring with an average temperature of 20°.9 (Table 1). Therefore, the difference in rate of development in respect to *Elodea* and to yeast is not in accord with the usual temperature effect. This effect was seen in stock females, of which the summer animals had an interovulatory period of 7.2 days (July) and in winter 12.2 days (January). Temperature may, however, have been effective in inducing significantly more rapid sexual development in the yeast-fed and *Elodea*-fed animals compared to that under constant *Elodea* feeding.

In relation to the effect of density upon reproductive activity it was found that the mean body size of females at the time of first oviposition was roughly inverse to the density (Fig. 4, *C*). The *Elodea*-fed animals showed the trend most clearly, but even on a yeast diet the difference between isolated and paired animals was significant ($P=0.024$). The antennal segment number, taken only in the yeast-*Elodea* experiment, was also inverse to density (Fig. 4, *D*). If antennal segment number is an index to instar, it is necessary to say that the 24 females in a density of 50 reached sexual activity at an earlier instar than the isolated animals, for the former possess on the average 20.9 segments and the latter 23.5. By reference to Table 2, the observed 20.9 segments would correspond to about instar 9, and the higher figure to instar 11 or 12. This result throws grave doubt on the validity of correlating antennal segments and instar stage, since a density as high as 50 animals, at least at the age of sexual maturity, has retarded all processes here studied.

Age at first sexual activity (Fig. 4, *B*) showed a tendency to increase with increasing density when the animals are fed upon *Elodea*. However, in the experiments in which yeast was used, sexual maturity on the average occurred most rapidly in population 4, the same density in which males had the largest size (Fig. 3). The accelerated maturity of density 4 over density 1 is significant in Yeast V ($P=0.003$).

The relation of fecundity to density is made difficult by the absence of a density 1 in *Elodea* I and the paucity of isolated females in Yeast I. In the latter experiment, however, the 12 paired females shed significantly more eggs than did the 5 isolated (Fig. 4, *A*; $P=0.010$). This is contrary to the correlation in stock females between body and brood size, for these more fecund paired females were smaller in size than the less fecund isolated animals. In *Elodea* I the increase in brood size of density 4 as compared to density 2 is insignificant ($P=0.84$), but the drop from density 4 to density 10 approaches significance ($P=0.09$). In consideration of the three curves (Fig. 4, *A*), with an increase in density above 4, an inverse relationship appeared between fecundity and density. Prior to that density the numbers present seemed to have little effect, at least when the natural food *Elodea* is included in the diet.

In conclusion of the section on reproductive activity it may be said that *Elodea* fed constantly from birth increased the size of the females when adult, the fecundity, and the rate of sexual development as compared to the effect of a yeast diet. The latter two aspects were also accelerated when *Elodea* was added to a yeast diet.

The females showed in general that body length and antennal segment number were in inverse relation to density. The mean age at first oviposition is youngest in population 4 of the animals fed yeast or those fed yeast and *Elodea*. The number of eggs per brood, apart from density 1, is not significantly affected by density, except on a yeast diet, until 6 or more amphipods are present. However, in the present state of our knowledge these indications cannot be taken too seriously.

SURVIVAL

General.—In order to obtain a basis for statistical analysis of survival, the time taken to reach 50 per cent survival was calculated for each population density of the eight experiments. This was converted to a percentage of the total time used by all densities of each experiment. On this percentage basis densities 1 and 2 were compared with densities 10 and 20; the latter were found to survive significantly longer ($P = 0.001$). Likewise, densities 10 through 100 were significantly more resistant than isolated animals and those of lower densities ($P = 0.005$).

The above generalization indicates the greater ability to survive of the individuals living in the higher densities. This generalization is substantiated in the results of the separate experiments which are presented in survivorship curves (Fig. 5). In these curves time—that is, age in days—is plotted on the abscissae and the number living on the ordinates. The latter figure is a percentage, since the number has been corrected to an initial seeding of 100 animals. In order to avoid confusion by the interlacing of curves only 5 population densities are given for each experiment. In *Elodea* II and III only 4 population densities were used (Table 1); therefore, those experiments are presented completely.

Populations fed upon Elodea.—Three facts were indicated by the first experiment (Fig. 5, *Elodea* I), which were supported by later observations. First, the animals in a very high density (50) survived best early in life, but, as they grew older or increased in size, maximal survival appeared and was maintained in an intermediate density (25). Secondly, populations did not always follow the general trend (density 5). Lastly, the decrease in numbers living was great at first among the animals of low densities and only later in the high densities; compare the curve of the paired animals and that of the density of 50.

Elodea II and III were alike in all particulars except that in *Elodea* III the water was never changed but merely passed through silk bolting cloth weekly. Both experiments were conducted at the same time. As in *Elodea* I, maximal survival shifted in *Elodea* II from a density of 50, and, since density 20 was out of line, density 10 survived maximally from the thirty-sixth day until the end of the counts on the fiftieth day. In *Elodea* III the animals of population 8 lived best from the beginning until the end. The greater death rate, in this experiment, of populations 20 and 50 was apparently correlated with the heavily conditioned, unchanged water. This result is atypical compared to those of the other experiments.

Populations fed upon yeast.—The type of food appeared to have no effect on the influence of density upon survival. In Yeast I (Fig. 5), as in *Elodea* I and II, a dense population (100) survived maximally at first, until the nineteenth day of age. Thereafter, density 20 best maintained its numbers until the end of the counts (32 days). After its initial maximal survival, the death rate in population 100 was so great that only 42 per cent were alive at the end, as compared to 56 per cent of the single animals or 63 per cent of density 50 (not graphed). In line with earlier results the deaths were initially most numerous among the isolated animals, after that the survivors maintained their numbers.

In Yeast II and III the death rate through all populations was very high for some unknown reason. However, in Yeast II, the maximal survival of an intermediate density (10) was demonstrated again, and also the minimal resistance of the isolated animals for at least the first month of age. Yeast III provided the only exception to the observation that isolated animals never survived maximally. In fact, so irregular was the survival of the populations that it was difficult to see a trend had it not been for preceding and fol-

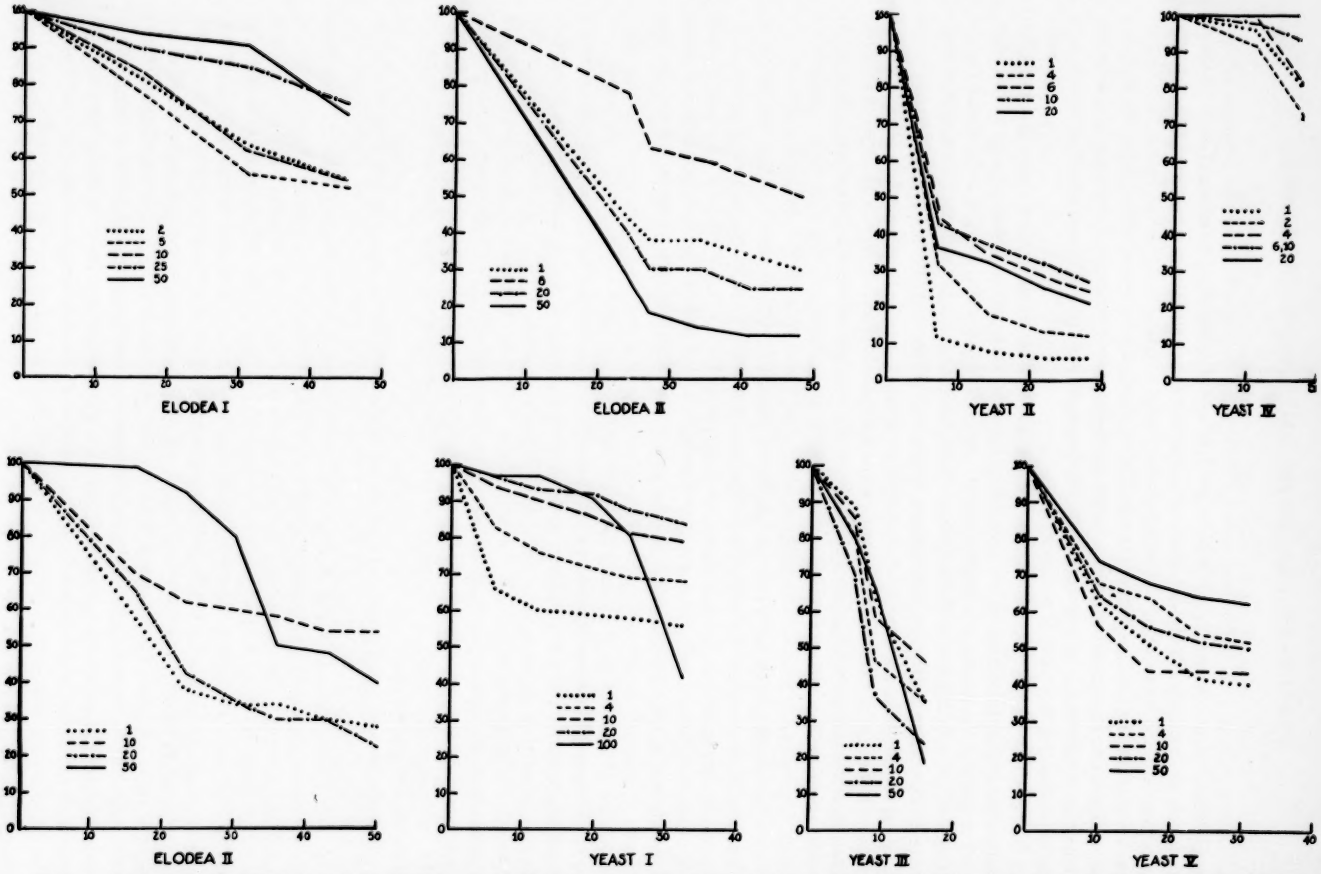

FIG. 5.—Percentage survival in the eight experiments. Ordinates, percentage alive; abscissae, time in days of age; inset key, population density plotted according to given symbols.

lowing experiments. Four days after the experiment had started, the deaths had been so numerous that replacement of animals had been made from the same stock of young originally used. In no other experiment had this been done; the results obtained may be influenced by that method.

In Yeast IV and V the highest density used—20 and 50, respectively—survived the best from beginning to end (Fig. 5), and no shift to a lower density occurred. Because of the use of chloretone, the measure of survival was restricted to 2 weeks and 1 month, respectively, in the two experiments.

In conclusion of this discussion of the effects of density upon the survival of the populations, it may be said that, although survival varied greatly from experiment to experiment and within experiments, nevertheless, a certain pattern in relation to density has emerged in nearly all cases. The type of food and water has not affected the results to an appreciable extent. With one exception (Yeast III, 6 days of age), maximal survival occurred in densities of 10 or above at every measured age. In respect to time the pattern tended to display a shift of survival from a high density to a lower (above 6), with the greatest death rate among the isolated animals and lowest densities in the first week or so of age, after which the death rate rose rapidly in the highest densities.

ENVIRONMENTAL FACTORS IN THE AMPHIPOD POPULATIONS AND DISCUSSION OF THEIR POSSIBLE EFFECTS

Increase in population density has both augmented and decreased the magnitude of physiological response in *H. azteca*. The effect has been dependent upon the particular process considered, the intensity of crowding, and, apparently, to some extent upon the conditions of the environment apart from density.

Density is a highly complex environmental factor and brings concomitant with it Smith's "density-dependent" factors of resistance (Bodenheimer, 1938), or, as expressed by Maclagen and Dunn (1936) "autobiotic factors of control." In the artificial microcosm of the experimental bottles containing the *Hyalella* populations these include directly proportional changes of carbon dioxide tension, other excretory products, fecal matter, collision frequency, and inversely proportional changes in oxygen, food, and space. Some of these have been measured (Table 6). The chemical analyses were made on water from populations seeded with adult males fed upon 4 drops of yeast. The tests started on the sixth day after seeding and ended on the eighth. No attempt has been made to measure the food consumed, but from the original distribution both food and space per animal may be estimated. Densities 50 and 100 seemed to clean up the yeast before the end of the week between feedings; however, there was apparently an excess of food at all times for the isolated animals. The 2 inches of *Elodea* appeared to provide an excess of food for all populations. However, it is realized that a quantitative excess may be qualitatively inadequate.

In Table 6 it may be seen that the 6 measured factors of the environment show a rough proportionality to the density with the exception of pH and conductivity. It is probable that the slight change in pH would have no effect, for the range is far less than that found to be effective on other organisms. The observation that both pH and conductivity dropped very slightly from density 1 to density 4 and then rose again is probably without significance, except in the fact that the two measures support each other. If significant, no explanation is at hand, or for the sudden increase in conductivity at density 50.

The question arises how these measured differentials, ingredients of the "density-dependent environment," may affect physiological processes in arthropods and whether these effects may separately or together have acted to produce the observed results on growth, reproduction, and survival of *H. azteca*.

The retardative effects of quantitative and qualitative malnutrition are too well established to need citation. In fact, von Dehn (1937) considered brood size an indication of the adequacy of the diet fed to cladocerans. In this respect the higher fecundity of the amphipods when fed throughout life upon *Elodea* as opposed to yeast is instructive, quite apart from the question of the population density. Using different diets Anderson (1930)

TABLE 6

ENVIRONMENTAL DIFFERENTIALS DEPENDENT UPON THE POPULATION DENSITY

Density	CO_2* (Cc.)	O_2† (Cc.)	Conductivity Mhos $\times 10^{-4}$	pH	Drops of Yeast per Animal	Space per Animal (Cc.)
1	52.2	5.10	2.97	7.67	4	100
2	52.6	4.96	2.97	7.60	2	50
4	52.7	4.87	2.96	7.57	1	25
6	53.1	4.67	2.97	7.60	0.67	16.7
10	54.2	4.28	3.01	7.70	0.40	10.0
20	57.4	4.06	3.09	7.70	0.20	5.0
50	66.2	3.70	3.71	7.67	0.08	2.0
Blank 1	49.8	5.61	2.75	7.83	100 cc. well water 7 days old	
Blank 2	52.5	5.53	2.85	7.80	The same with 4 drops of yeast	
Blank 3	55.3	3.95	2.96	7.63	The same with 8 drops of yeast	

* Cubic centimeters CO_2 included that given off by animals, atmospheric, and chiefly that derived from the breakdown of all carbonates and bicarbonates in the water. For the sum of the latter two factors see Blank 1.

† Both gas analyses were made by the Van Slyke method, values are the average of two readings, on the sixth and seventh days, respectively.

found that on a poor diet isolated female *Daphnia* matured later and passed through more instars prior to the release of young. Sexual maturity occurred, he stated, at a given body size rather than at a constant instar. The irregularity of sexual maturity of the isolated control amphipods fed upon a pure yeast diet may correlate with this observation. However, it is in contradiction to results from the experimental populations in which the sexually mature females of the higher population densities (less food per animal) were smaller in size and of a lower antennal segment number than those in less crowded densities (Fig. 4). A lipoid factor, resembling vitamin E, appears to be necessary for maximal reproductive expression in *Gammarus* (O'Brien and Yarnold, 1937). The role of undernutrition associated with crowding was said to account in part for the decreased fecundity of *Drosophila* since many flies disturbed one another while feeding (Pearl, 1932).

The factor of restricted space decreased fecundity of lepidopterans, but the effect was less drastic than that of undernutrition (Hofmann, 1933). Space itself is not a simple factor but involves proportional changes in other factors and cannot, therefore, be used

per se without very careful analysis, despite attempts to consider it of psychological importance.

The increase in some unstable excretory products coincident with crowding is believed to cause the production of males in cladoceran cultures. By this result a depressant action is ascribed to these substances, since artificial depression of metabolism by chemical agents or by low temperature occasioned the appearance of males under conditions otherwise suited to the production of female offspring (Banta, 1937). Carbon dioxide alone did not increase male production in Cladocera (Banta, 1937), and, on the whole, there are few data concerning its action within normal range. In the tests on water from amphipod populations no distinction was made between free and bound carbon dioxide, and it is only the former which would be effective. If pH is an indication of free carbon dioxide, there is much less differential throughout the populations than the analysis for the gas would suggest. Oxygen tension might be effective in producing a proportional metabolic rate if oxygen consumption was dependent upon oxygen tension. This is, however, rarely the case over relatively normal fluctuations of tension. The results on studies of Crustacea allow no generalization (Hyman, 1929).

On the whole, from the few examples given above it can be concluded that at least undernutrition and accumulating excretory products might be responsible for the decrease of fecundity and development at and beyond a density threshold, of which the level would be determined by the susceptibility of the particular process and physiological condition of the organism. At extreme densities one or both might increase the death rate. The rapid death rate of the 100 population at an advanced age (therefore size) could be so explained. The low survival of populations 20 and 50 in *Elodea* III (Fig. 5) when the water was never changed support this view.

No evidence has thus far been presented in explanation of the continued maximal survival and early maximal growth of animals in intermediate densities. Survival at least may be related by analogy to the greater resistance of animals of lowered metabolic rate to adverse elements in the environment in accordance with the work of Child (Fowler, 1931). Evidence is also present that massed animals or their products may ameliorate a toxic or deficient environment (Allee, 1931; 1938). Such an explanation may also be applied to the results on growth and fecundity, with the added complication that in this case the yeast diet must be partially deficient, for the animals fed upon *Elodea* grew inversely to the density, while only those fed upon yeast displayed a positive density effect. The diminution of this effect on growth with increasing age may be related to the increasing strength of the density-dependent factors of resistance as the animals become larger.

Allee (1938) presents the retardative and augmentative effects of density in two curves in order to avoid the use of the terms "harmful" and "beneficial." Magnitude of physiological response is plotted on the ordinates and numbers of animals on the abscissae. The first curve (Type *A*) is monophasic, showing an inverse relationship; Type *B* is diphasic with an initially direct relationship which changes to an inverse one. The former curve could be entirely the result of density-dependent factors of resistance which increase in direct relation to crowding. The diphasic curve could be produced by the interaction of two sets of resistances, one of which would act inversely, the other directly in proportion to the density. A toxic or inadequate medium which could be ameliorated or neutralized by the products of density might be considered a resistance inverse to density, for it would retard most strongly the isolated animals. The same products of density increasing beyond the point of neutralization would form the second set of resistances, acting direct-

ly in relation to numbers of animals present. These resistances, whether they act directly with or inversely to density, are examples of density-dependent factors. At the population density in which the sum of the two sets of resistances is least, maximal physiological response would be expressed.

SUMMARY

1. *Hyalella azteca* Saussure has been used to demonstrate the effects of population density upon growth, reproduction, and survival. All populations were contained in 100 cc. of either city water (Lake Michigan), with or without chlorine, or well water and fed upon equal amounts of *Elodea* stem or yeast. The experimental animals were grown under these conditions from a few days after release from the maternal brood pouch until at least 1 month of age. The densities used ranged from 1 to 100 amphipods.

2. When fed upon *Elodea* the animals grew, at least after the second week of age, inversely to the density of the population. The adult females were of a body length inverse to density, as were those fed upon yeast supplemented after 1 month by *Elodea*. In populations fed upon yeast, on the other hand, mean maximal body or head length occurred initially in the animals of a high density, but with increasing age (or size) the maximum shifted to lower densities. Sexually mature males of density 4 were the largest, but the mean body length of females, at the time of first oviposition, was greatest in the isolated animals.

3. On a diet including *Elodea* fecundity appeared to be little affected by density until 6 or more animals were present; beyond this density fecundity was inverse to numbers present. However, on a yeast diet the females of population density 2 were significantly more fecund than those isolated, and the females of population density 4 when fed either yeast or yeast and *Elodea* released the first brood earlier than those from more or from less crowded populations.

4. Survival of the animals varied greatly from experiment to experiment and also in respect to density. The greater survival, however, of animals in populations of 10 or over was substantiated in all eight experiments, and was not related, in any apparent way, to either the type of water or the type of food. As in the results upon growth of yeast-fed populations, an initially maximal survival in a high density (100 or 50) tended to drop to a lower density, but never below 8, with advancing age. This shift was occasioned by a late but very rapid death rate in the highest densities. In the isolated animals or low densities there was initially a high death rate, which decreased as the animals grew older.

5. Analyses of the water containing populations of 1–50 adult males demonstrated a direct proportionality between density and carbon dioxide tension, and an inverse relation between oxygen tension, food, and space. There was little effect on pH. Conductivity changed only slightly until a density of 50 showed a disproportionally great increase.

6. The above stated relation between density and carbon dioxide, presumably other excretory products, food, and space might well be responsible for inhibition of growth and fecundity and, at very high densities, of the ability to survive. It is suggested that the accelerated growth and fecundity of yeast-fed animals and the greater resistance of all animals in an intermediate density may be related to the heightened resistance caused by lowered metabolism or to the amelioration of a toxic or inadequate environment by the coincident products of density.

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THE UNIVERSITY OF CHICAGO PRESS
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Physiological Zoölogy is published quarterly by the University of Chicago at the University Press, 5750 Ellis Avenue, Chicago, Ill. The subscription price is $7.50 a year; the price of single copies is $2.25. Orders for service of less than a full year will be charged at the single-copy rate. Postage is prepaid by the publishers on all orders from the United States, Mexico, Cuba, Puerto Rico, Panama Canal Zone, Republic of Panama, Dominican Republic, Canary Islands, El Salvador, Argentina, Bolivia, Brazil, Colombia, Chile, Costa Rica, Ecuador, Guatemala, Honduras, Nicaragua, Peru, Hayti, Uruguay, Paraguay, Hawaiian Islands, Philippine Islands, Guam, Samoan Islands, Balearic Islands, Spain, and Venezuela. Postage is charged extra as follows: for Canada and Newfoundland, 20 cents on annual subscriptions (total $7.70), on single copies 5 cents (total $2.30); for all other countries in the Postal Union, 48 cents on annual subscriptions (total $7.98), on single copies 12 cents (total $2.37). Patrons are requested to make all remittances payable to The University of Chicago Press in postal or express money orders or bank drafts.

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Claims for missing numbers should be made within the month following the regular month of publication. The publishers expect to supply missing numbers free only when losses have been sustained in transit and when the reserve stock will permit.

Business correspondence should be addressed to The University of Chicago Press, Chicago, Ill.

Communications for the editors and manuscripts should be addressed to W. C. Allee, Managing Editor of PHYSIOLOGICAL ZOÖLOGY, The University of Chicago, Chicago, Ill.

The articles in this journal are indexed in the *International Index to Periodicals*, New York, N.Y., the *Quarterly Cumulative Index Medicus* and *Biological Abstracts*.

Applications for permission to quote from this journal should be addressed to The University of Chicago Press, and will be freely granted.

Entered as second-class matter October 27, 1928, at the post-office at Chicago, Illinois, under the Act of March 3, 1879.

[PRINTED IN U.S.A.]

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